

THE DEVELOPMENT OF CLASS-SPECIFIC AND COMPOUND
SPECIFIC ANTIBODIES FOR THE DETECTION, IDENTIFICATION
AND EXPOSURE MONITORING OF GENOTOXIC CARCINOGENS

by

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To

Dinah and Eleanor

ABSTRACT

Mercapturic acids are urinary metabolites which signal exposure to genotoxic compounds. Carefully designed hapten-protein conjugates and a judicious screening strategy has enabled the generation of compound and class-specific antibodies which bind mercapturic acids.

S-phenylmercapturic acid (S-PMA) is a highly specific and sensitive marker of benzene exposure. A monoclonal antibody reactive with S-PMA has been generated. The immobilised antibody retains immunoreactivity and can be used to enrich S-PMA from the urine of benzene exposed workers. The performance of the immunoaffinity column has been validated by comparison with data obtained from GC/MS analysis of urine from benzene exposed workers (range 12-168ug/l. corr. coeff. 0.98, n=23). Furthermore immunoaffinity chromatography facilitates the quantitative determination of urinary S-PMA by HPLC. Bioconcentration of S-PMA from the urine of benzene exposed workers has permitted the quantification of S-PMA by HPLC at 8 hour Time Weighted Average exposures of around 1ppm.

Monoclonal antibodies to low molecular weight mercapturic acids (eg. S-(2-hydroxyethyl)mercapturic acid) were generally of too low affinity for practical application. As an alternative approach antibodies to adducted protein were investigated. Antibody 4D3 binds the adducted N-terminal heptapeptide released from the alpha-chain of haemoglobin by trypsin hydrolysis. Initial studies suggest antibody 4D3 can bind the adducted heptapeptide in whole haemoglobin. This may facilitate the determination of adducted haemoglobin in biomonitoring studies.

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ABBREVIATIONS

ABTS	2,2-azino-di-(3 ethylbenz-thiozoline sulphonic acid)
ABS	(aminobenzoyloxy)succinimide
Ac	acetylated
aq	aqueous
Bala	beta-alanine
BS ³	bis(sulfosuccinimidyl)suberate
BSA	bovine serum albumin
7C	7-carbon spacer arm
CNBr	cyanogen bromide activated sepharose
CI	chemical ionisation
Ci	curries
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxynucleic acid
DNP	dinitrophenol
dpm	disintergrations per minute
ECD	electrochemical detection
EDC	N-ethyl-N-(3-dimethylamino-propyl)-carbodiimide
EI	electron ionisation
ELISA	enzyme linked immunosorbent assay
FAB	fast atom bombardment
FAC's	flourescent activated cell sorter
FCS	foetal calf serum
Gel	gelatin
GC	gas chromatography
GPR	general purpose reagent
Hb	haemoglobin
HCl	hydrochloric acid
2-HEMA	S-(2-hydroxyethyl)mercapturic acid
HSA	horse serum albumin
Ig	immunoglobulin
2-GEMA	S-(2-guanylethyl)mercapturic acid
GSH	glutathione
HAT	hypoxanthine, aminopterin, thymidine
HPLC	high performance liquid chromatography
HGPRT	hypoxanthine guanine phosporibosyl transferase
HT	hypoxanthine, thymidine
KLH	keyhole limpet haemocyanin
LET	low energy transmission
M	molar
MA	mercapturic acid
MEL	maximum exposure limit
MES	2-(N-morpholino)-ethanesulphonic acid
n	sample number
min	minute
MS	mass spectroscopy
MSA	mouse serum albumin
MW	molecular weight
NHS	N-hydroxysuccinimide
NMR	nuclear magnetic resonance
OD	optical density
OHEt	hydroxyethyl

PEG	polyethylene glycol
PBS	phosphate buffered saline
PFPIITC	pentafluorophenyl isothiocyanate
PFPTH	pentafluorophenylthiohydantoin
PEL	permissible exposure limit
ppm	parts per million
Rf	flow rate ratio
RIA	radioimmunoassay
rpm	revolutions per minute
SRBC's	sheep red blood cells
S-BMA	S-benzylmercapturic acid
Sp2's	Sp2/O.Ag8 myeloma cell line
S-PMA	S-phenylmercapturic acid
SDS	sodiumdodecylsulphate
SIM	selective ion monitoring
THF	tetrahydrofuran
TK	thymidine kinase
TLC	thin layer chromatography
TNBS	trinitrobenzenesulfonic acid
TRIS	Trizma base
TWA	time weighted average
UV	ultra violet

CHAPTER ONE

THE DEVELOPMENT OF COMPOUND AND CHEMICAL CLASS-SPECIFIC
ANTIBODIES FOR THE DETECTION, IDENTIFICATION AND
EXPOSURE MONITORING OF GENOTOXIC CARCINOGENS

INTRODUCTION

1.1 GENOTOXICITY

There is little doubt that chemists have contributed to the improved quality of human life by the synthesis of a whole host of industrial, agricultural and medicinal compounds. However, human exposure to certain chemicals remains hazardous. A correlation between chemical exposure and cancer incidence in humans was first recognised some 200 years ago in 1775 by Percival Potts. Potts observed that the occurrence of scrotal cancer in chimney sweeps appeared to be related to their exposure to soot and tar. Today, 522 agents have been shown to be carcinogenic in animal studies and 55 compounds are associated with the formation of cancers in humans (Ashby and Paton 1992).

It is now generally accepted that most chemical carcinogens and probably all chemicals that induce cancer *de novo*, ie. cancer initiators, operate via a mutational or genotoxic mechanism (Wright, 1991). DNA is the key (primary and critical) target of the genotoxic carcinogens - the essential requirement being the possession of a centre(s) of electrophilic reactivity which permits chemical reactions with nucleophilic centres in the target molecule. In many instances this electrophilic reactivity is conferred on unreactive precursor molecules by metabolic activation in exposed organisms (Miller 1978, Miller and Miller 1981). The primary products formed during the reactions between genotoxic chemicals and DNA are generally promutagenic (or lethal), and their occurrence leads to an increased risk of mutation and cancer. The detection of these products, therefore, signals exposure to a genotoxic carcinogen(s)

and their chemical characterisation and measurement provides a basis for identifying and estimating the doses of ultimate carcinogens delivered to their critical target (target dose) - essential for proper risk assessment. At present it is thought that there is no absolutely safe exposure dose and that the initiating actions of genotoxic chemicals (cancer initiation, genetic disorders) are additive and cumulative with no operational threshold in the low dose range (Wright 1990, Britton et al 1991). Among these products, DNA adducts have been singled out for special attention, not only because of their mutagenic or cancer initiating properties, but also because of the high sensitivity, specificity and resolving power of the methods available for their analysis.

The measurement of DNA adducts in tissues probably provides the most direct and satisfactory basis for detecting, identifying and measuring critical exposures to ultimate genotoxic agents. However, tissue DNA is not readily available for such monitoring purposes in human populations and a surrogate dose monitor is required. In addition to reactions undergone with DNA, genotoxic chemicals also react with nucleophilic centres in protein molecules giving rise to the corresponding range of protein adducts. The reactivity of genotoxic chemicals for proteins has led to the use of readily accessible blood proteins (eg. the haemoglobin of circulating erythrocytes) as surrogates for tissue DNA to monitor human exposures to known and previously unknown or undetected hazards. (For a review see Ehrenberg, 1991).

In addition to reactions with informational macromolecules, electrophiles also undergo spontaneous and enzyme-catalyzed

reactions with the sulphhydryl group of glutathione to form adducts (or conjugates). In the main, such reactions represent an effective detoxification step leading ultimately to the excretion of the electrophile in the form of the corresponding mercapturic acid conjugate in the urine.

In general, and in quantitative terms, the production of glutathione adducts far exceeds reactions with both DNA and proteins. Consequently, urinary mercapturic acids provide a valuable source of material for the detection, identification and monitoring of exposures to genotoxic chemicals. However, the analysis of mercapturic acids, particularly the initial preparative work prior to assay, are complex and lack the desired sensitivity. Monoclonal antibodies can be used in an immunoaffinity column to isolate small quantities of specific chemicals from complex matrices as an aid to quantification of these chemicals by physico-chemical methods.

Immunoaffinity chromatography is a form of adsorption chromatography in which the molecule to be purified is specifically and reversibly adsorbed by an antibody molecule (ligand) covalently attached to an insoluble support (matrix). The compound to be purified is applied in a solution to the column bed and is selectively adsorbed by the immobilised antibody. After washing away unbound material, the affinity complex is dissociated and the purified compound eluted.

A major aim of this project was to develop single step immunoextraction procedures to facilitate and improve the qualitative and quantitative analysis of both known and currently unknown mercapturic acids.

1.2 GLUTATHIONE

The tripeptide glutathione, γ -L-glutamyl-L-cysteinylglycine, (Fig. 1.1) discovered in the 1920's by Sir F. G. Hopkins, has been a subject of intensive study. It is the commonest intracellular thiol and is present in bacterial, plant and animal cells in high concentrations. In mammalian cells, for example, the intracellular level of glutathione is in the range of 0.5-10mM. Glutathione functions through its glutamyl bond and SH group in many biological processes including metabolism, transport and cellular protection (Larsson 1983, Meister and Anderson 1983).

Glutathione is synthesized in the liver in two stages (Figure 1.2). First, the formation of a glutamyl linkage between L-glutamate and L-cysteine is catalyzed by glutamyl-cysteine synthetase. This is followed by the addition of glycine to the glutamyl-cysteine molecule catalyzed by glutathione synthetase. Over 99% of the intracellular glutathione is in the reduced form GSH, and it is GSH which is actively transported from the liver to other organs (Griffith and Meister 1979 and Meister 1983).

Breakdown of GSH also occurs intracellularly (Figure 1.2). Glutamyl transpeptidase initiates GSH degradation either by transpeptidation, in which glutamyl is transferred to a peptide acceptor, auto-transpeptidation or hydrolysis. Synthesis, transport and catabolism of GSH form the basis of the glutamyl cycle.

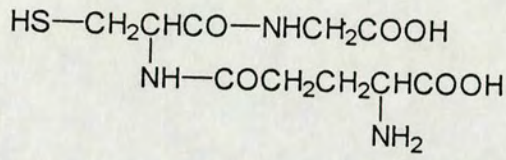


Figure 1.1 Glutathione

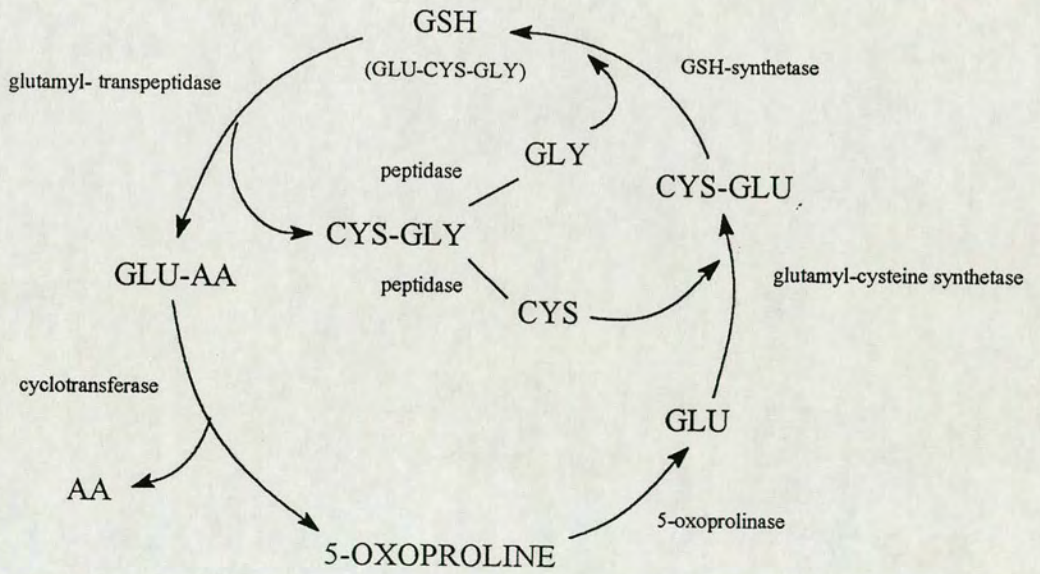


Figure 1.2 The Glutamyl Cycle

The glutamyl cycle plays an important role in the cellular uptake of glutamyl amino acids, and GSH appears to be the storage and transport form of the amino acid cysteine.

GSH itself plays a major role in cellular oxidation-reduction reactions. Under conditions of oxidative stress GSH can be reversibly oxidized, either enzymatically or non-enzymatically, leading to an increase in the disulfide (GSSG).

Cells are continuously exposed to a range of oxidative insults - both hydrogen peroxide and free oxygen radicals are the products of many normal cellular reactions. Free radicals also result from exposure to ionizing radiation, or from the administration of certain drugs.

GSH functions in other reductive processes essential for cellular metabolism including protein synthesis, protein degradation, and in alternative pathways of ribonucleotide reduction.

GSH is known to function as a cofactor in several enzyme systems. It is a cofactor in the glyoxalase system which eliminates cellular keto-aldehydes. Keto-aldehydes inhibit cell proliferation and this may explain how cellular thiols play a role in the control of cell proliferation.

GSH levels decrease with age and falling levels may play a part in cell ageing. GSH may also be involved in autoimmune disease, the incidence of which increases with age. GSH levels are known to vary in patients with diabetes, and some forms of diabetes are considered to be autoimmune diseases.

1.3 GLUTATHIONE TRANSFERASES

The excretion of foreign compounds as N-acetylcysteine derivatives, the mercapturic acids, was first noted in 1879 (Baumann et al) but it took many years before glutathione was shown to be the source of cysteine. Nakashima et al (1934) reported naphthalene depleted GSH in the liver cells and eye lenses of rabbits, and Yamamoto et al (1944) demonstrated that a fall in liver GSH when rabbits were dosed with bromobenzene was related to an increase in mercapturic acid excretion. Barnes et al (1959) observed the same in rats for a number of different mercapturic acid precursors, and showed that the rate of GSH turnover in the liver was sufficient to sustain mercapturic biosynthesis.

It was Booth (1961) who confirmed the presence of a liver enzyme which could catalyze the conjugation of foreign compounds with glutathione. Investigations soon demonstrated many different GSH-S transferases. These enzymes were shown to catalyze more than one reaction and were initially classified according to the type of substrate involved (eg. GSH S-aryltransferase, GSH S-epoxidetransferase, GSH S-alkyltransferase, GSH S-alkenetransferase, GSH S-aralkyltransferase) (Chasseud 1979).

It is now known that these enzymes possess a broad and overlapping substrate specificity, and a key factor in the reaction of the substrates appears to be the electrophilicity of the carbon atom which the GSH attacks. By contrast, GSH S-transferases have an almost absolute specificity for GSH as the thiol substrate. The GSH S-transferases appear to act by bringing the electrophilic substrate

into close proximity with GSH thereby stopping its reaction with other cellular nucleophiles. GS^- is the reacting nucleophile and GSH S-transferases may promote ionization of the GSH sulfhydryl group, thereby increasing its nucleophilicity (Keen et al 1976). Many of the reactions catalyzed by the GSH S-transferases can also proceed non-enzymatically, and in some cases non-enzymatic reactions occur so rapidly under physiological conditions that enzyme catalysis is difficult to demonstrate.

The most studied GSH S-transferases are soluble forms found in the cytosol, mitochondria and nucleoplasm, but membrane bound forms exist in the mitochondria and endoplasmic reticulum (Kraus and Gross 1979). The highest concentrations can be found in the hepatic tissues and other tissues that come into contact with high concentrations of xenobiotics or their metabolic products. In the liver (rat and human) 2-10% of the extractable proteins are GSH S-transferases.

1.4 MERCAPTURIC ACIDS

Glutathione S-conjugates are converted to mercapturic acids (Fig.

1.3) by three enzyme catalyzed reactions (Jakoby 1980):

1. The removal of the glutamyl moiety catalysed by glutamyl transpeptidase.
2. Removal of the glycine moiety from the glycine-cysteine molecule by a number of potential aminopeptidases and dipeptidases.
3. N-acetylation of the cysteine conjugate, a reaction catalyzed by N-acetyltransferase, to yield a mercapturic acid.

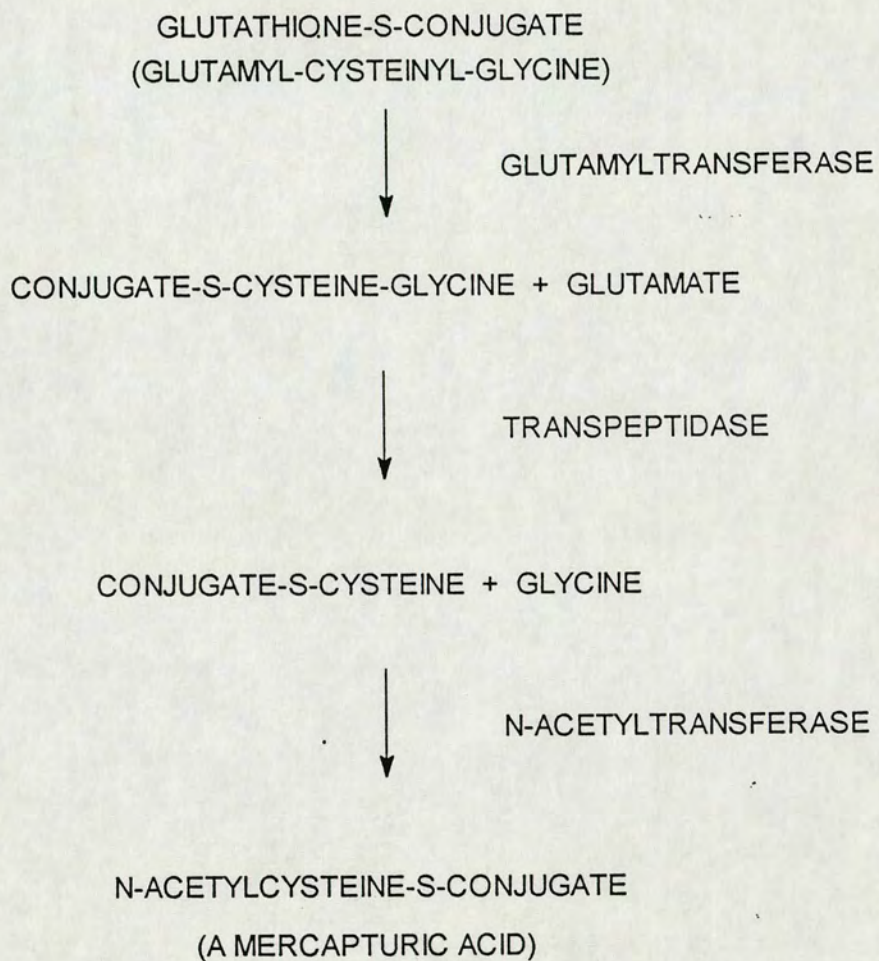


Figure 1.3 Metabolism of glutathione conjugates to mercapturic acids

In mammalian studies the highest levels of glutamyl transpeptidase, aminopeptidase/dipeptidase and N-acetyltransferase activity have been found in the kidney. The mercapturic acids are subsequently excreted in the urine.

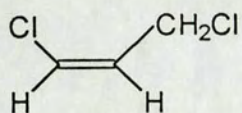
1.5 EXPOSURE MONITORING

Methods for monitoring exposure to potential mutagens and carcinogens fall into two distinct groups - (1) the measurement of the substance, its metabolites, and its reaction products with cellular macromolecules, and (2) the quantification of a biological endpoint which may be a result of an organism's exposure (Van Sittert 1985). The determination of urinary thioethers (eg. Van Sittert 1987) can be included in the former. Quantification of urinary metabolites can give a more accurate measurement of the absorption of a substance than can be achieved from the estimation of the agent in the environment. Urinary metabolites are a measure of the internal dose. It should be noted, however, that measurement of urinary thioethers only determines the proportion of the ultimate carcinogen/mutagen excreted in the urine after detoxification. It does not measure the proportion of the dose which evades detoxification and reacts with cellular macromolecules. The proportion of the dose reacting with cellular macromolecules is the effective dose. A knowledge of compound metabolism is therefore needed.

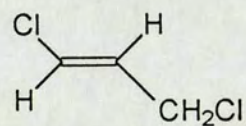
1.6 MERCAPTURIC ACIDS AND BIOMONITORING

The assay of urinary thioether detoxification products as a tool in detecting human exposure to electrophilic compounds was first reviewed in 1981 by Van Doorn et al. Van Doorn demonstrated that spectrophotometric analysis of urinary mercapturic acids, after extraction and alkaline hydrolysis had an important signalling function - that is, an increase in urinary mercapturic acids indicated an exposure to one or more electrophilic compounds. However, the assay of urinary thioethers is non-selective, the compounds causing the raised levels remain unidentified, and Van Doorn concluded that more selective methods were to be preferred.

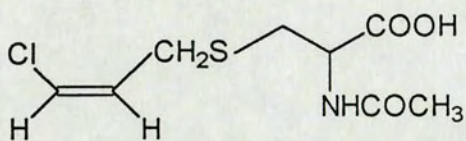
Onkenhout et al (1986) outlined 3 GC/MS methods for the specific detection of N-acetyl-S-(Z or E 3-chloropropenyl-2)(-L-)cysteine (Fig. 1.4) - the mercapturic acid excreted after exposure to Z and E 1,3-dichloropropene. Dichloropropene is a major constituent of a number of commercial soil fumigants and a known mutagen (Watson et al 1987). Applicators in the Dutch flower bulb industry are exposed to 1,3-dichloropropene and the respiratory occupational exposure to the Z and E isomers of 1,3-dichloropropene has recently been assessed (Brower et al 1991). Van Welie et al (1991) have shown that the Z and E mercapturic acids of 1,3-dichloropropene can be measured in the urine of exposed flower culture workers at levels suitable for use in a biological monitoring programme.



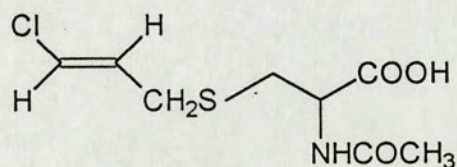
Z-1,3-DICHLOROPROPENE



E-1,3-DICHLOROPROPENE



Z-DCP-MERCAPTURIC ACID



E-DCP-MERCAPTURIC ACID

Figure 1.4 Conjugation of Z and E dichloropropene

1.7 BENZENE EXPOSURE

Benzene is an important industrial chemical. In the past it was used as a solvent in the manufacture of paint, plastic and rubber products. Today it is the starting material for the synthesis of polystyrene and other bulk chemicals. Benzene enriched aromatics are added to gasoline as replacements for alkyl lead compounds due to their anti-knock properties. Benzene is emitted in engine exhaust gases, cigarette smoke, and is ubiquitous in the environment.

Benzene possesses appreciable vapour pressure and hazardous occupational exposure due to inhalation can occur. Petrochemical workers, petrol station attendants and cigarette smokers are at risk as chronically exposed populations (Yardley-Jones et al. 1991). A person with respiratory rate of 16/minute and a tidal volume of 0.5l will absorb 7.5ul of benzene per one hour through the lungs in an environment of 10 ppm (Nelson 1992).

The major effect of chronic benzene exposure is its haemopoietic toxicity, unique to benzene among the simple aromatic hydrocarbons. The hallmark of benzene exposure is pancytopenia, a decrease in the number of circulating erythrocytes, leukocytes and thrombocytes. Continued exposure may lead to aplastic anaemia and leukemia (Snyder et al. 1985). The occupational exposure limit in the United Kingdom (Maximum Exposure Limit, MEL) and the United States (Permissible Exposure Limit, PEL) have recently been lowered to 5ppm and 1ppm, 8 hour TWA (Time Weighted Average) respectively, reflecting a concern for the risk of neoplasia. Benzene is a Group 1 carcinogen.

1.8 BENZENE METABOLISM

Studies indicate that one or more metabolites of benzene mediate haemopoietic toxicity. Early studies identified phenol, trans,trans-muconic acid, phenylmercapturic acid, ethereal sulfate and glucuronide conjugates of catechol, hydroquinone and trihydroxybenzene as metabolites of benzene (Snyder et al. 1985). Following the synthesis of pure C¹⁴ benzene Parke and Williams (1952) were able to account for all of an oral dose of benzene in rabbits. Over a 2 day period 43% of the administered benzene was exhaled unchanged, 1.5% was exhaled as carbon dioxide and 35% was excreted in the urine, mainly as conjugated phenols. Acid hydrolysis of the conjugates showed phenol (23%) was the major component, the minor components were hydroquinone (4.8%), pyrocatechol (2.2%), trans,trans-muconic acid (1.3%), phenylmercapturic acid (0.5%) and hydroxyhydroquinone (0.3%). The rest of the benzene remained in the body to be eliminated more slowly in the urine or as carbon dioxide in expired air. The metabolism of benzene appears to be similar in humans and rabbits.

Benzene metabolism takes place predominantly in the liver but also in the bone marrow (Fig. 1.5). Benzene is first metabolised to benzene epoxide by mixed function oxidases. Benzene epoxide is converted non-enzymatically to phenol, or enzymatically to either a premercapturic acid derivative or benzene dihydrodiol. Phenol and dihydrodiol can be further metabolised to hydroquinone and pyrocatechol respectively (Yardley-Jones et al 1991).

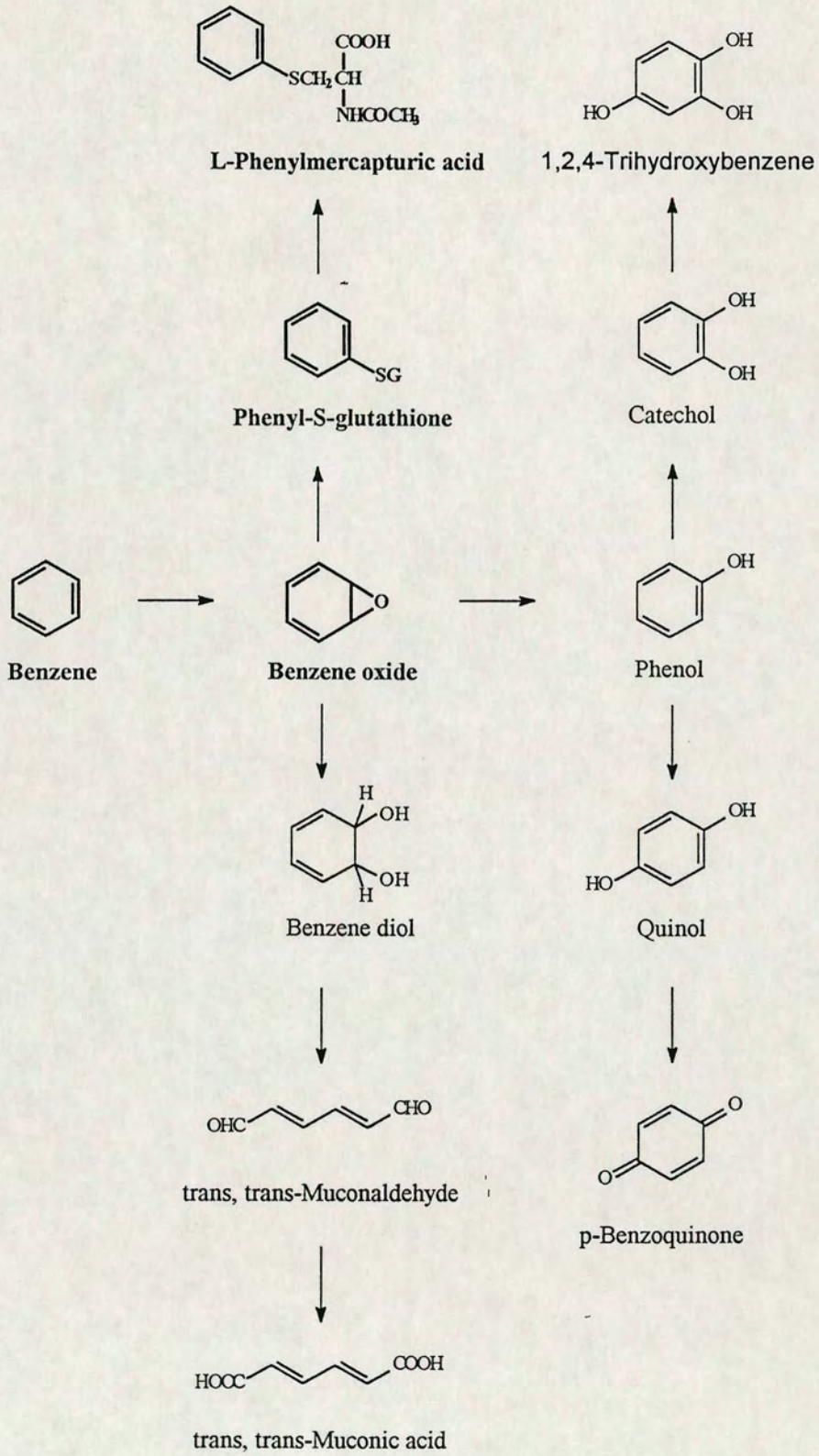


Figure 1.5 Benzene metabolism

1.9 BIOMONITORING BENZENE EXPOSURE

Benzene in blood and exhaled air has been used as a specific and sensitive biomarker for measuring occupational and non-occupational exposure to benzene (Perbellini et al. 1988). For occupational exposure monitoring non-invasive sampling methods are preferred. Several urinary metabolites of benzene have been examined as potential biomarkers of occupational exposure.

Urinary phenol is suitable for monitoring exposures, 8 hours TWA, of 10ppm or greater (Van Haaften and Sie 1965). At exposures of less than 6ppm it is not reliable due to a lack of specificity. There is a linear relationship between benzene exposure and urinary phenol concentration. Haaften compared gas chromatography procedures with two colorimetric methods for determining phenol concentrations, and found the former procedure to be superior in terms of sensitivity.

Recently, urinary trans, trans-muconic acid has been proposed as a biomarker of benzene exposure (Inoue et al. 1989). Trans, trans-muconic acid can be directly determined in a urine sample after precipitates have been removed by centrifugation. There is a linear correlation between concentration of urinary trans, trans-muconic acid and TWA benzene exposure measured in the breath zone of workers. It is possible to separate groups of workers exposed to 6 ppm of benzene from non-exposed populations. The increased sensitivity of muconic acid over phenol determinations is due primarily to the low background levels of muconic acid. Trans, trans-muconic acid allows the determination of exposures down to 1ppm 8 hours TWA (Boogaard and Van Sittert 1995). Trans,trans-

muconic acid levels in non-exposed populations are below the detection limit of the assay (less than 0.1 mg/ml). Trans, trans-muconic acid is not suitable for individual exposure monitoring because 95% confidence limits on individual values fall between 25-50ppm. An additional drawback is that coexposure to toluene lowers urinary trans, trans-muconic acid concentrations. Benzene is often combined with toluene in solvent preparations.

1.10 S-PHENYLMERCAPTURIC ACID

S-phenylmercapturic acid is a highly specific marker of benzene exposure (Stommel et al 1989). Boogaard and Van Sittert (1995) have demonstrated that S-phenylmercapturic acid could be used as an indicator of benzene exposures of 0.3 ppm at the group level. It may also possibly be used at the individual level. Stommel found a linear correlation between benzene exposures of 1-100 ppm and urinary S-phenylmercapturic acid in the rat. Above 100ppm excretion is proportionately less.

To determine the most appropriate sampling time, Van Sittert et al (1993) studied the pattern of S-PMA excretion, and found that mercapturic acid was excreted in a single phase in most workers, the highest concentrations being detected at the end of a shift (average half-life of elimination 9 ± 4.5 hours). In some workers, the highest levels were found at the beginning of the next shift. This delayed excretion could be due to skin exposure to benzene or to individual differences in toxicokinetics. In some workers a tentative second phase of elimination was found with a half-life of

45 +/- 4 hours. The average S-phenylmercapturic acid concentration in end of shift samples from workers exposed to 1 ppm of benzene for 8 hours is 46ug/g creatinine (approximately 100ug/l).

1.11 S-(2-HYDROXYETHYL)ACETYL-L-CYSTEINE

Gerin and Tardif (1986) measured urinary S-(2-hydroxyethyl)acetyl-L-cysteine in rats after exposure to ethylene oxide, and indicated a possible biological monitoring role for this mercapturic acid in man. S-(2-hydroxyethyl)mercapturic acid (2-HEMA) has also been shown to be a urinary metabolite of several other important industrial chemicals including vinyl chloride (Watenabe et al 1976), 1,2-dibromoethane (Van Bladeren et al 1980), 2-bromoethanol (Jones and Wells 1981) and acrylonitrile (Van Bladeren et al 1981). A critical evaluation of the possibilities and limitations of 2-HEMA as a potential tool in biological monitoring studies has been promoted by Vermeulen et al (1989).

1.12 BIOTRANSFORMATION REACTIONS LEADING TO S-(2-HYDROXYETHYL)ACETYL-L-CYSTEINE

A number of possible biotransformation reactions may lead to the excretion of 2-HEMA (Vermeulen et al 1989).

The halohydrins are widely used industrial chemicals which have no known carcinogenic potential but possess high acute toxicity. Urinary excretion of 2-HEMA has been reported for the halohydrins 2-bromo and 2-iodoethanol. Three possible biotransformation reactions may lead to the excretion of 2-HEMA. These are 1) the direct conjugation of the compound with GSH, 2) its metabolism to ethylene

oxide and its subsequent conjugation to GSH, and 3) conversion of the halohydrin to a haloacetaldehyde by the enzyme ethanol dehydrogenase, conjugation of the haloacetaldehyde with glutathione followed by reduction of the aldehyde group to the alcohol (Fig. 1.7).

The 1,2-dihaloethanes are also widely used industrial chemicals (solvents, pesticides, fumigants, and gasoline additives) which have been associated with carcinogenic effects in animal studies. 2-HEMA has been identified as a urinary metabolite of rats treated with 1,2-dibromomethane. Three metabolic pathways which, in principle, may lead to the excretion of 2-HEMA have been derived for the 1,2-dihaloethanes. These metabolic pathways proceed through the formation of haloethanol and/or haloacetaldehyde, episulphonium ion or halonium ion intermediates (Fig. 1.8).

Apart from the halogen substituted ethanes, other disubstituted ethanes may give rise to 2-HEMA via GSH dependent metabolic pathways. For example, the alkylating ester ethylene dimethane sulphonate (Fig. 1.9) and chloroethylamine derived compounds (such as the anti-neoplastic agents ribopyranosyl-1-nitrosourea, RPCNU, and cyclophosphamide, Fig. 1.10). The later appear to be metabolized via an oxidative N-dealkylation reaction to chloroacetaldehyde and/or chloroethanol. Chloroacetaldehyde and chloroethanol are compounds which may directly, or via an intermediate chloroethylcarbocation, give rise to 2-HEMA (Fig. 1.11).

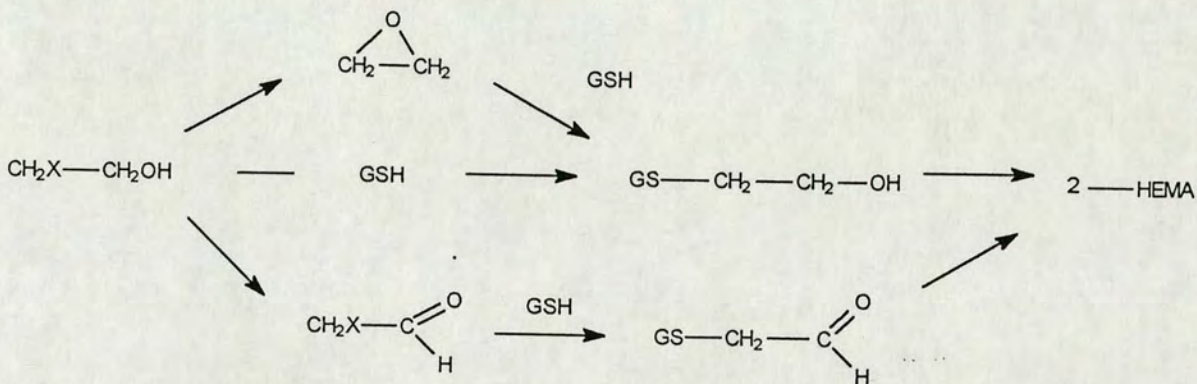


Figure 1.7 Possible biotransformation reactions of halohydrins leading to the excretion of 2-HEMA.

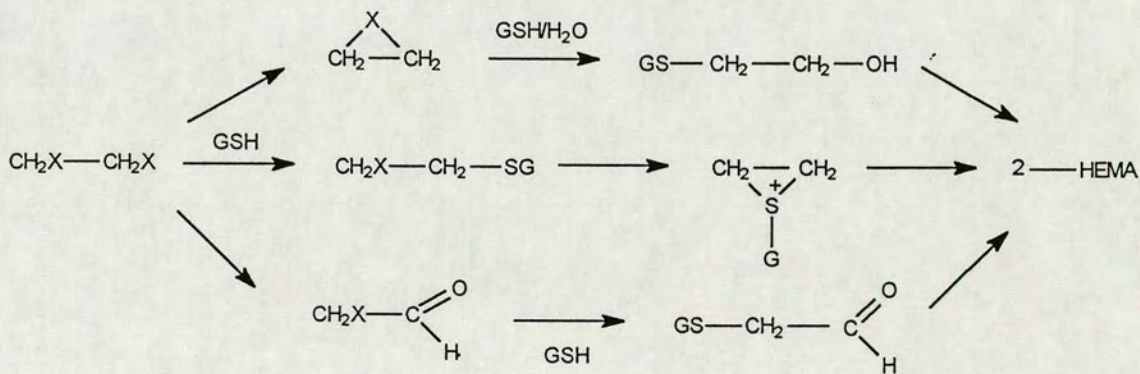


Figure 1.8 Possible biotransformation reactions of the 1,2-dihaloethanes leading to 2-HEMA excretion

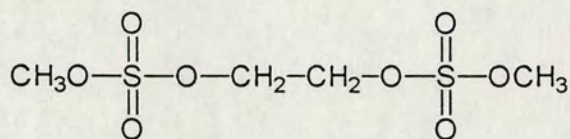


Figure 1.9 Ethylene dimethane sulphate

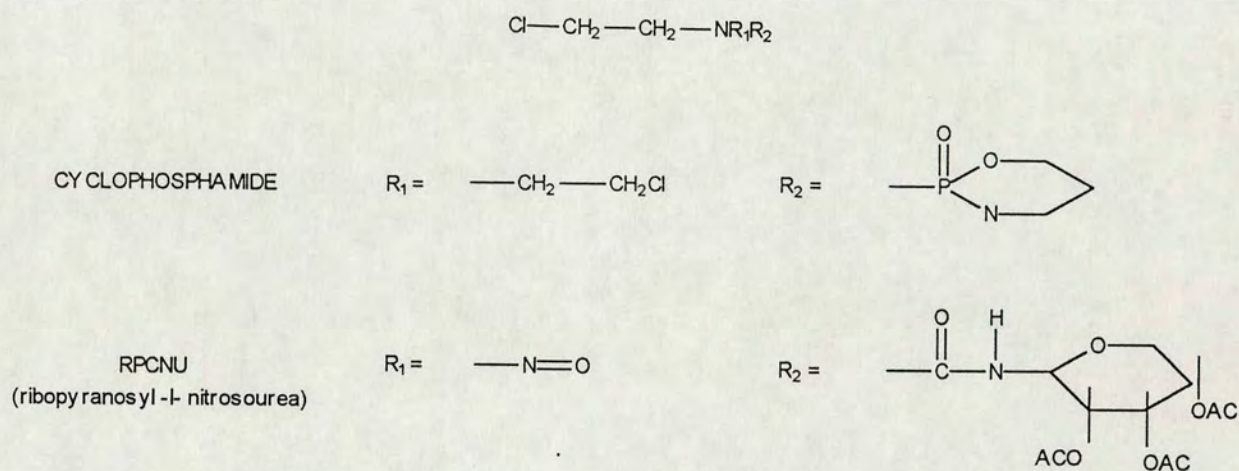


Figure 1.10 RPCNU and cyclophosphamide - chloroethylamine derived compounds

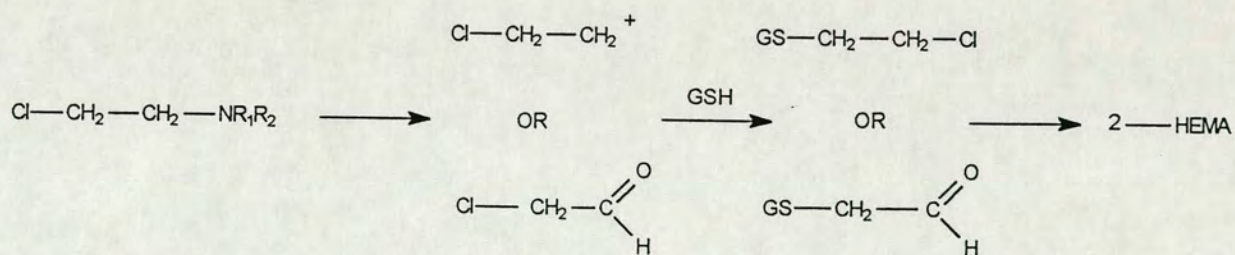


Figure 1.11 Formation of 2-HEMA from chloroethylamine derived compounds

Ethylene is an important industrial chemical. It is used in the manufacture of plastics, as a plant growth regulator and is a component of diesel exhaust and tobacco smoke. Ethylene itself is relatively non-toxic but can be metabolized by cytochrome P450 in the liver to ethylene oxide. Ethylene oxide is a known mutagen and carcinogen. Conjugation of ethylene oxide with glutathione can lead to the excretion of 2-HEMA (Fig. 1.12).

Ethylene derived compounds may also give rise to 2-HEMA. The mono-substituted ethylene vinyl chloride is a known carcinogen widely used in the polymer industry. Vinyl chloride is metabolized via a primary epoxidation to an oxirane. This can rearrange to form an haloacetaldehyde which, as seen above, can be conjugated by GSH and subsequently excreted as 2-HEMA (Fig 1.13).

The monomer acrylonitrile (vinyl cyanide) is used in the manufacture of rubber, plastics and adhesives. It is a carcinogen, teratogen and a potent toxin of the central nervous system. It is highly reactive and may act as a direct alkylating reagent. There are two major routes of biotransformation. A direct non-enzymatic conjugation with GSH leads to the excretion of the mercapturic acid N-acetyl-S-(2-cyanoethyl)-L-cysteine. Indirectly, vinyl cyanide can be oxidised to an epoxide which may be conjugated by GSH and excreted as 2-HEMA. In rats, both mercapturic acids are excreted as urinary metabolites (Fig. 1.14).

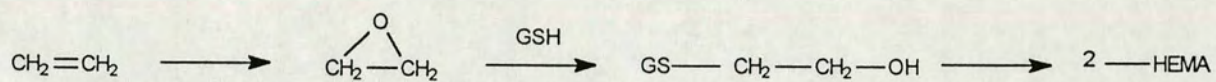


Figure 1.12 Conjugation of ethylene oxide

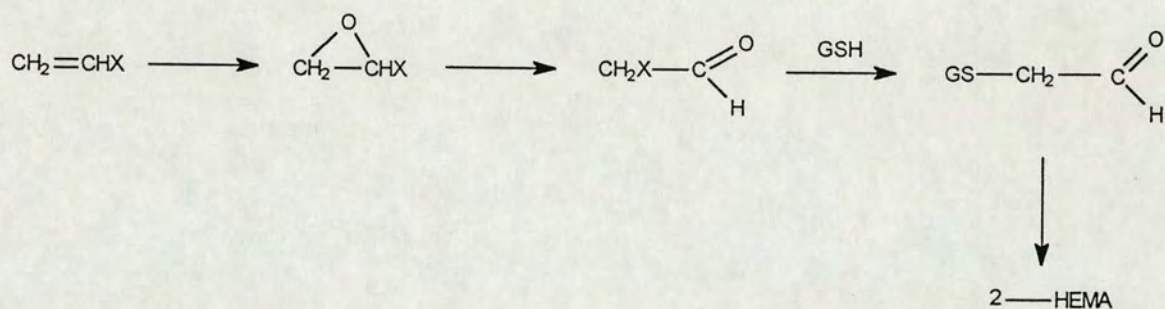


Figure 1.13 Metabolism of ethylene vinyl chloride

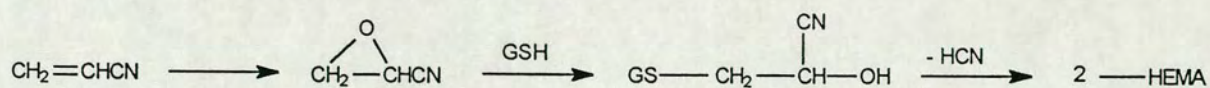


Figure 1.14 Metabolism of acrylonitrile

1.13 BIOACTIVATION AND GLUTATHIONE TOXICITY

Until recently glutathione conjugation was recognised as a universal detoxification mechanism. Bedford et al (1975) was the first to deduce that reaction with glutathione can, in certain circumstances, give rise to intermediates which are very reactive towards nucleophilic attack. Bedford proposed that 2-HEMA could be produced from ethylene bromide via a reactive episulphonium ion - this has subsequently been confirmed. It is now accepted that glutathione conjugation is an important bioactivation mechanism with implications for organ-selective toxicity and carcinogenicity. Glutathione-dependent toxicity has been recently reviewed by Anders et al (1992).

The vicinal dihaloethane 1,2-dibromoethane is a widely used industrial chemical with known mutagenic and carcinogenic properties. The major urinary metabolite of 1,2-dibromoethane in rats is 2-HEMA. Up to 50% of a single oral dose (500µmol/kg body weight) is excreted as 2-HEMA (Van Bladeren et al 1980). Two possible biotransformations may lead to 2-HEMA excretion - 1) the direct conjugation of 1,2-dibromoethane with glutathione which gives rise to a thiiranium ion, and 2) oxidation of 1,2-dibromoethane to bromoacetaldehyde, conjugation of bromoacetaldehyde and glutathione followed by the reduction of the aldehyde group to yield a 2-hydroxyethyl-glutathione conjugate. 2-hydroxyethyl-glutathione is metabolised to 2-HEMA. In rats the direct conjugation and oxidative routes occur in the ratio 1:4 (Van Bladeren et al 1981b). The thiiranium ion is able to bind covalently to DNA to give (2-(N-7-guanyl)ethyl)glutathione. In rats exposed to 1,2-dibromoethane 95%

of the DNA adducts formed were S-(2-(N-7-guanyl)ethyl)glutathione. This adduct is excreted in the urine as N-acetyl-S-(2-(N-7-guanyl)ethyl)-L-cysteine (2-GEMA). The urinary excretion of 2-HEMA, 2-GEMA, and the formation of DNA adducts in the liver and kidney were dose dependent. 2-GEMA may be considered to be the first mercapturic acid acting as a biomarker of effective dose. However, to be applicable in exposure monitoring the sensitivity of 2-GEMA analysis must be increased considerably (Van Welie et al 1992).

Some haloalkenes (eg. hexachlorobutadiene, tetrachloroethene and chlorofluoroethene) are known nephrotoxins, nephrocarcinogens, or both. The selective nephrotoxicity of these compounds appears to be due to a multistep pathway involving hepatic glutathione S-conjugate formation. The glutathione conjugates are transported to the kidney where they are metabolised to the corresponding mercapturic acid. The mercapturic acids are converted to reactive thiol conjugates by cysteine conjugate beta-lyases. The thiol conjugates may be further transformed to a number of generally unknown reactive sulphur intermediates (Fig. 1.15).

1.14 REACTIVE INTERMEDIATES LEADING TO S-(2-HYDROXYETHYL)ACETYL-L-CYSTEINE

The biotransformation reactions leading to 2-HEMA may proceed by five reactive intermediates (thiiranium ion, activated double bonds, (halo)ethylcarbonium ions, (halo)oxiranes and (halo)acetaldehydes). It is difficult, however, to translate the occurrence of certain

reactive intermediates into a potential type or intensity of toxicity (Vermeulen et al 1989). Since toxicity also depends upon the rates of formation and degradation of the intermediate, intermolecular interactions and any organ or tissue selective features.

Reactive intermediates have been classified according to the "hard" and "soft" acid-base properties of electrophiles and nucleophiles (Coles 1985). "Hard" electrophiles and nucleophiles possess ionic character, they have isolated charges, small atomic radii, low polarizability and a low ease of oxidation. "Soft" electrophiles and nucleophiles have covalent character, a low positive charge, a large size, filled outer orbitals and a high polarizability. Comparing electrophiles and nucleophiles, as a general rule "hard" reacts with "hard" and "soft" reacts with "soft". Nucleophilic sites in proteins (cysteinyll and lysinyll amino groups) are "soft", and the nucleophilic sites in DNA (ring and exocyclic nitrogen and oxygen atoms) tend to be "hard". "Soft" electrophiles such as cyano-containing compounds and haloacetaldehydes have been shown to form covalent adducts with cysteinyll residues in proteins, and "harder" electrophiles such as the thiiranium ion have been shown to form adducts with "harder" nucleophiles present in DNA (Vermeulen et al 1989).

Intermediary compounds such as ethylene oxide and some halooxiranes give rise both to protein and DNA adducts (Fig. 1.16).

POTENTIALLY ELECTROPHILIC CHEMICALS

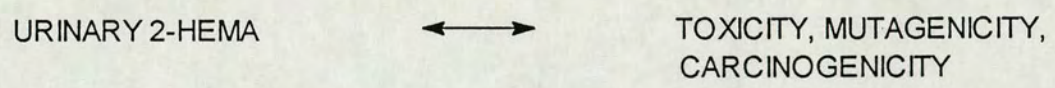
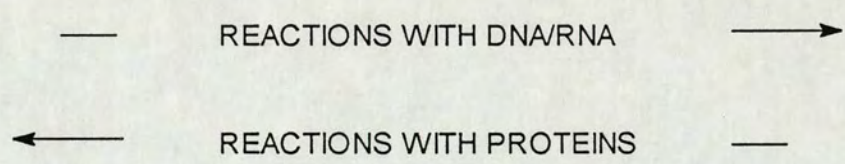
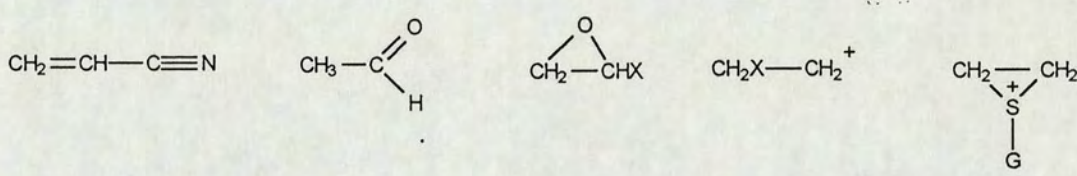


Figure 1.16 Intermediates in the formation of 2-HEMA

Measurement of mercapturic acids is therefore important for two reasons - in the bioinactivation of electrophilic compounds and in the bioactivation of electrophilic intermediates.

1.15 ANALYTICAL PROCEDURES FOR MERCAPTURIC ACIDS

Several techniques have been described for the qualitative and quantitative analysis of the mercapturic acids. These include thin-layer chromatography, spectrophotometry, amino acid analysis, HPLC and GC. In general the assay of the mercapturic acids can be divided into three steps - isolation, derivatisation and analysis.

Mercapturic acids can be extracted from acidified urine (pH 2 or less) using organic solvents such as ethyl acetate. Because of the high polarity of mercapturic acids such as 2-HEMA, solid-liquid extraction (ie. freeze drying followed by redissolution of the residue in a methanol-ether mixture) may yield higher recoveries. For application at low urinary concentrations the latter procedure has to be preferred (Vermeulen et al 1989).

The most selective and sensitive analytical procedures for the quantitative analysis of urinary mercapturic acids employ HPLC and GC separation techniques. Separation of mercapturic acids can be achieved by reverse phase chromatography on C8-18 silica, cation and anion exchange columns.

Due to the low volatility and high polarity of mercapturic acids, S-PMA and 2-HEMA must be derivitized prior to GC analysis. Separation can be achieved on apolar or medium polar columns. Flame-ionization,

nitrogen-phosphorus, sulfur-selective detection and mass spectrometry have all been used to detect mercapturic acids after GC. GC/MS is the most sensitive procedure for the detection of mercapturic acids.

At present only GC/MS possesses the required sensitivity for biomonitoring occupational exposure to benzene (0.1ug/l). Assays for 2-HEMA lack sensitivity for biomonitoring. Background levels of 2-HEMA cannot be identified in human urine. A naturally occurring background is expected because of the presence of endogenous hydroxyethylating agents, such as ethylene. For application in occupational monitoring assay sensitivity should be at least 0.1mg/ml (van Welie et al 1992).

1.16 PROJECT AIMS - THE COMPOUND AND CLASS-SPECIFIC IMMUNOPURIFICATION OF MERCAPTURIC ACIDS

A limitation of GC/MS analysis, for both biomarkers, is a complex work-up prior to assay. The sensitivity of the procedures is also limited by the purity of the samples. The development of single step extraction procedures may both facilitate and improve the sensitivity of the quantitative MS analysis of S-PMA and 2-HEMA. In addition, GC/MS analysis restricts this method to use in sophisticated laboratories. The use of an immunoaffinity column to enrich S-PMA and 2-HEMA may permit the analysis of mercapturic acids by HPLC which at present lacks the sensitivity for application in biomonitoring programmes. The major aim of this project was to develop immunoenrichment systems for S-PMA and 2-HEMA.

Current immunopurification systems are generally compound specific, i.e. each designed to isolate one specific analyte. However, a broader specificity, which would permit isolation to be based on membership of a particular group or class of chemicals, could be even more useful. In biomedical monitoring studies a class-specific antibody which recognises mercapturic acids would greatly facilitate the identification of unknowns and permit simultaneous monitoring of mixed exposures.

The principle of class-specific immunopurification has been demonstrated in this laboratory. An immunoaffinity chromatography column has been prepared using a monoclonal antibody raised to 6-(2,4-dinitroanilino)hexanoic acid which selectively binds the 2,4-DNP moiety when the substituent at C1 of the aromatic ring is linked through an oxygen or a methylene carbon. This column has been used to selectively abstract chemicals containing the 2,4-dinitrophenyl group from an aqueous cocktail containing low concentrations of related chemicals variously substituted in the aromatic ring with one or two amino groups. Moreover, the immunopurification system has also proved effective in the selective and quantitative abstraction of these chemicals from the complex matrix urine.

A second aim of this project was to develop class specific antibodies for application in the detection and identification of mercapturic acids to facilitate the monitoring of exposures to genotoxic carcinogens.

CHAPTER TWO

SYNTHESIS AND CHARACTERISATION OF MERCAPTURIC ACIDS,
MERCAPTURIC ACID-PROTEIN CONJUGATES AND THE INDUCTION OF ANTIBODIES
WHICH BIND MERCAPTURIC ACIDS IN MICE

INTRODUCTION

2.1 THE IMMUNE RESPONSE

The invasion of a vertebrate's body by a foreign substance elicits an immune response. Part of this response is the secretion of antibodies by plasma cells. Antibodies contain binding sites which recognise the shapes of determinants (epitopes) on the foreign substance. Antibody-epitope binding induces a number of processes which eventually lead to the neutralisation and elimination of the foreign substance from the host. A typical response is highly heterogeneous. Many different antibodies are produced which recognise different determinants or the same determinant with varying affinities. Each antibody is secreted by a different lymphocyte. If a lymphocyte could be grown in culture then a large quantity of identical antibody could be produced. Unfortunately lymphocytes cannot be maintained in culture.

A myeloma is a tumour of antibody secreting cells which is derived from a single cell and secretes a single type of antibody. Myeloma cells can be grown indefinitely in culture medium, but against which determinant they are directed remains unknown. Attempts to induce myelomas of known specificity have failed.

In 1975 Kohler and Milstein fused mouse lymphocytes from the spleen of an animal immunised with a known immunogen with myeloma cells. The resulting hybrid-myelomas, or hybridomas, had two important properties. They secreted antibody of known specificity and possessed immortal character. The hybridoma cells were cloned and single clones were grown in culture. The culture medium contained

antibodies of one type, all identical in structure and function. Kohler and Milstein had developed a powerful technique for dissecting out a polyclonal immune response into its monoclonal components. They were awarded a Nobel Prize for Medicine and Physiology in 1975 for this work.

2.2 HAPTEN IMMUNOCHEMISTRY

Compounds of low molecular weight (<1000) are not normally antigenic. S-PMA and 2-HEMA, the mercapturic acids of interest in this project, have molecular weights of 239 and 220 respectively. However, low molecular weight substances (haptens) can be rendered immunogenic by coupling to larger carrier proteins. Hapten immunochemistry was first investigated by Landsteiner in 1917. Landsteiner held an almost dogmatic belief that a special chemical constitution peculiar to proteins was required for antibody production. We now know this is not true but Landsteiner's work lay the foundation of modern immunochemistry.

Landsteiner prepared his hapten-protein conjugates either by the acylation of amino groups with acid chlorides and anhydrides, or by the diazotisation of the histidine, tyrosine and tryptophan amino acids. With these conjugates Landsteiner established that the original specificity of the protein carrier was altered and antibodies were produced by compounds which themselves were not antigenic. He also noted that antibodies were produced to the protein carrier and, to be certain that the antibodies were against the determinant group, it was necessary to test the sera against conjugates made with an unrelated protein.

A number of protein carriers have been used to stimulate antibody production. These include globulin fractions, the serum albumin of various species, haemocyanin, ovalbumin, thyroglobulin and fibrinogen. Whether choice of carrier significantly influences the antihapten response is a controversial subject. KLH is the preferred carrier in this laboratory and is known to induce a large immune response in immunised mice.

In his early experiments Landsteiner sought to determine the number of haptens coupled to protein which gave the optimal response. He concluded that too much or too little led to a poor response, and for serum albumin carriers ten haptenic groups appeared optimal. Niswender and Midgley (1970) studied steroid-protein conjugates and suggested a minimum of twenty molecules of hapten should be coupled to BSA, any less giving an inferior antigen. Klaus and Cross (1974), in studies on BSA-DNP conjugates, obtained a good response with five to nineteen DNP groups per BSA. However, fifty to sixty DNP's per BSA elicited an IgM response only. The nature of the hapten probably exerts a very strong influence on the response, but good antibody titres can usually be obtained with epitope densities between eight and twenty five.

Landsteiner also established in his work that antibody specificity is directed primarily at that part of the hapten molecule furthest from the functional group that is linked to the protein. Many other similar studies exist in the literature. This specificity can be further improved by the use of conjugates in which the hapten is attached via a spacer arm to the protein.

Regardless of the protein carrier used, the same functional groups are available for attachment to the hapten - the carboxyl group of the C-terminal and of the aspartic and glutamic acid residues; the amino groups of the N-terminal and the lysine residues; the imidazole and phenolic functions of the histidine and tyrosine residues; and the sulfhydryl group of cysteine. All have been used for the preparation of immunogenic hapten protein conjugates.

2.3 MERCAPTURIC ACID SYNTHESIS

S-PMA is commercially available, 2-HEMA is not commercially available and must be synthesized. Mercapturic acids have been successfully synthesised using three different strategies. These are:-

1. The reaction of the nucleophilic thiolate anion of N-acetyl-L-cysteine with the appropriate electrophile under alkali conditions (Van Bladeren et al, 1980) (Figure 2.1a).
2. The reaction of electrophilic sulfur in the sulfonylhalogenides and thiolsulfinates with an appropriate nucleophile (Buijs et al, 1986) (Figure 2.1b).
3. The reaction of a thiol with 2-acetamidoacrylic acid in a Michael type addition (Van Bladeren et al, 1980 and Buijs et al, 1986)(Figure 2.1c).

2-(hydroxyethyl)mercapturic acid has been synthesised by procedures 1 and 2 with yields of up to 82% (Van Bladeren et al, 1980). Purification of 2-(hydroxyethyl)mercapturic acid from the reaction mixture however is difficult due to its excellent solubility in aqueous systems.

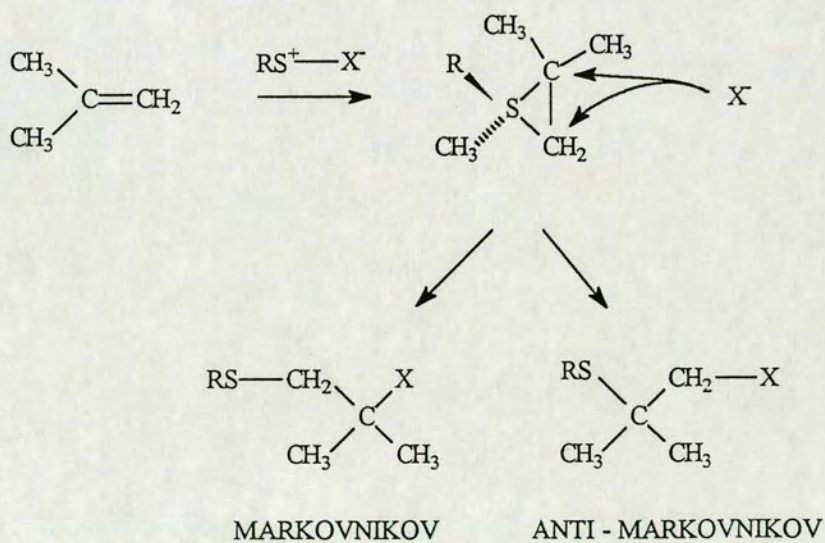
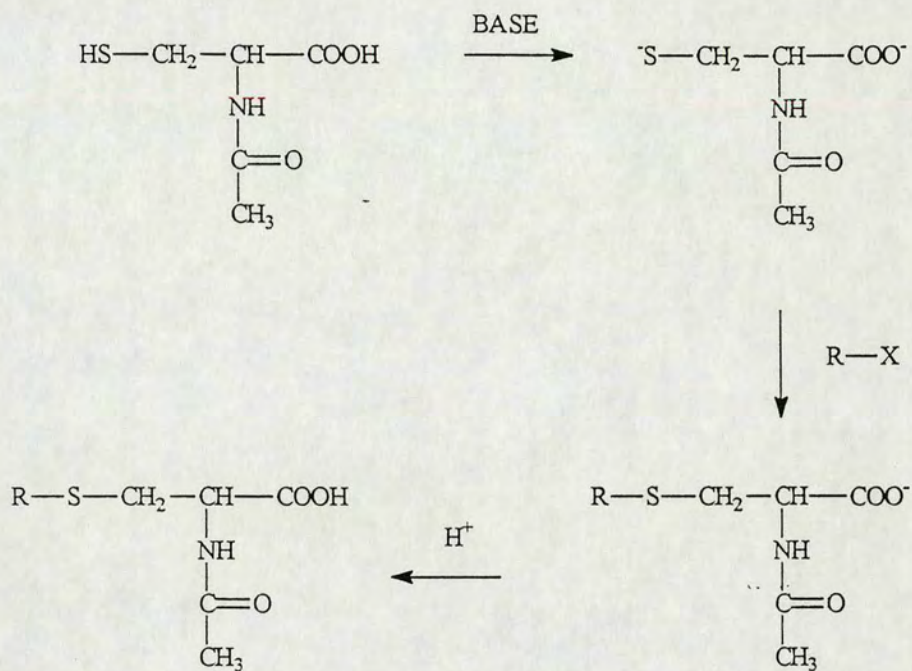
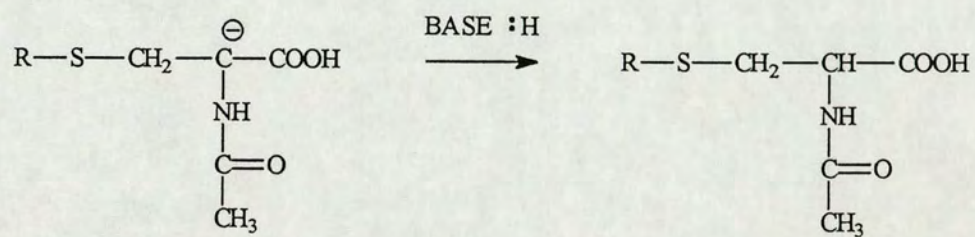
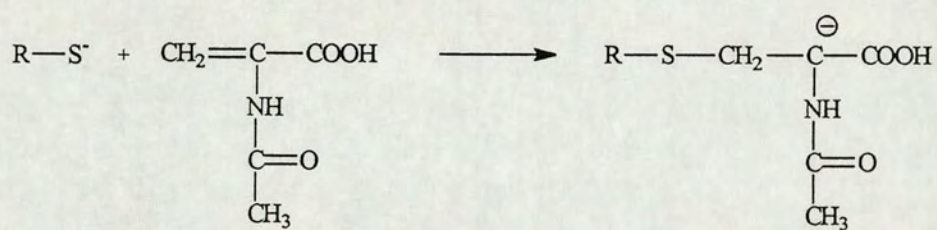
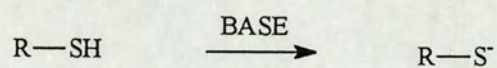


FIGURE 2.1 Synthesis of mercapturic acids a) by the reaction of the thiolate anion with an electrophile: b) by the reaction of electrophilic sulphur with a nucleophile:



and c) by a Michael type addition.

In his systematic study of mercapturic acid preparation, Van Bladeren found dry methanol to be suitable for the synthesis of 2-(hydroxyethyl)mercapturic acid. With sodium methoxide as the base (2 equivalents), N-acetyl-L-cysteine (dianion) was reacted with hydroxyethyl bromide (Figure 2.2). When the reaction was complete the solvent was either evaporated, the products taken up in acetone and neutralised with a minimum amount of concentrated HCl, or the solvent was neutralised with dry methanolic hydrogen chloride, evaporated and the residue resuspended in acetone. The salt was removed by filtration and, after evaporation of the solvent at reduced pressure, 2-(hydroxyethyl)acetylcysteine was obtained as a colourless oil which was recrystallised from chloroform/ether or ethylacetate/petroleum ether. The procedure avoids losses due to difficult extraction steps, and the presence of 2-(hydroxyethyl)mercapturic acids was confirmed by NMR and MS. Proper NMR and MS spectra however do not guarantee a pure reference standard, and similarly titration of the carboxylic acid groups with sodium hydroxide may give erroneously high yields due to interferences with N-acetyl-L-cysteine or alkali salts (Vermeulen et al, 1989).

Not all mercapturic acids can be synthesised by taking advantage of the nucleophilic properties of N-acetyl-L-cysteine in basic solution (Buijs et al 1986). An alternative synthetic route was devised by Buijs who employed the electrophilic properties of sulfur in sulfenylhalides. This strategy has been successfully employed to synthesise a number of mercapturic acids, and is particularly useful in the synthesis of mercapturic acids derived from the conjugation

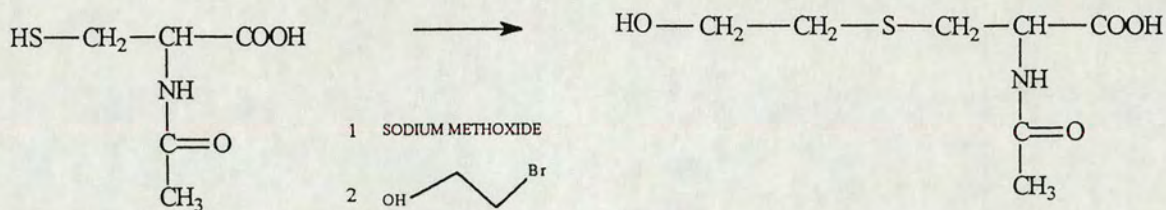


Figure 2.2 Synthesis of 2-HEMA by the method of Van Bladeren et al (1980).

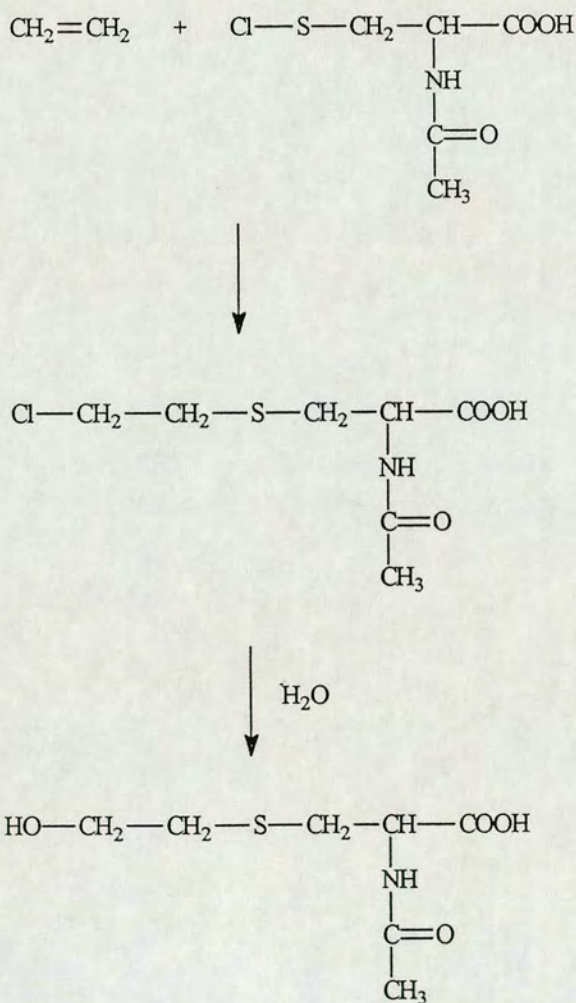


Figure 2.3 Synthesis of 2-HEMA by the method of Buijs et al (1986)

of glutathione with vicinal dihalogenoalkanes and dihalogenoalkenes which are difficult by other methods.

Buijs recommends the use of sulfenyl chlorides and bromides because sulfenyl fluorides and iodides are difficult to handle (N-chlorosuccinimide is the preferred chlorinating agent as no side reactions take place). 2-(hydroxyethyl)mercapturic acid may be synthesised by this route. First 2-(chloroethyl) and 2-(bromoethyl) acetylcysteine are synthesised by reacting the corresponding N-acetyl-L-cysteine derivative with ethene (yields of 79% and 86% respectively have been reported). These reactive β -halogeno thioesters are easily converted to 2-(hydroxyethyl) mercapturic acid by the addition of water (Figure 2.3).

The mechanism of addition of sulfenyl halides to alkenes is biphilic and can lead to both Markovnikov and anti-Markovnikov products. Aliphatic sulfenyl halides predominantly give Markovnikov products (ie. sulfur containing residue at the least substituted carbon), and anti-Markovnikov products tend to be observed only with sterically hindered alkenes. This is an important consideration since N-acetyl-L-cysteine is optically active and reactions with chiral or prochiral substrates only give mixtures of distereoisomers which may have different chemical or biological properties (figure 2.1b).

2.4 HAPTEN - PROTEIN CONJUGATION

A) CONJUGATION WITH CARBODIIMIDES

Sheehan and Hess (1955) introduced carbodiimides for the synthesis of peptide bonds from amines and carboxylic acids. The preparation

procedure is simple and easy to perform and it has become an important coupling method. The reaction may be represented as a dehydration whose mechanism is not fully understood but is thought to occur via an O-acylisourea intermediate (Figure 2.4). The formation of O-acylisourea is rapid and may lead to a peptide either by immediate aminolysis (i) or via a symmetrical anhydride (ii) with the production of urea. Urea is sparingly soluble in most solvents and can be easily removed by filtration. The intermediates are highly reactive and side reactions can occur. One such side reaction is the collapse of the O-acylisourea by intermolecular acyltransfer which competes with the desired attack by external nucleophiles, and leads to the formation of the less reactive N-acylurea (Figure 2.4).

Acylureas are the main side products at elevated temperatures and, in order to shift the reaction toward peptide bond formation, temperatures around 0°C should be used. Dicyclohexylcarbodiimide is the most commonly used carbodiimide. It is soluble only in organic solvents. Dicyclohexylurea formed in coupling reactions with dicyclohexylcarbodiimide is insoluble in most solvents. It precipitates out during coupling reactions and can be easily removed by filtration. 1-ethyl-3-(dimethylamino)carbodiimide is a popular water soluble carbodiimide. The urea formed during coupling with this reagent is water soluble and is removed by dialysis when the conjugation of hapten to protein is complete.

Problems can occur when using carbodiimides as the coupling reagent, for example rapid hydrolysis of the reactive O-acylurea can lead to

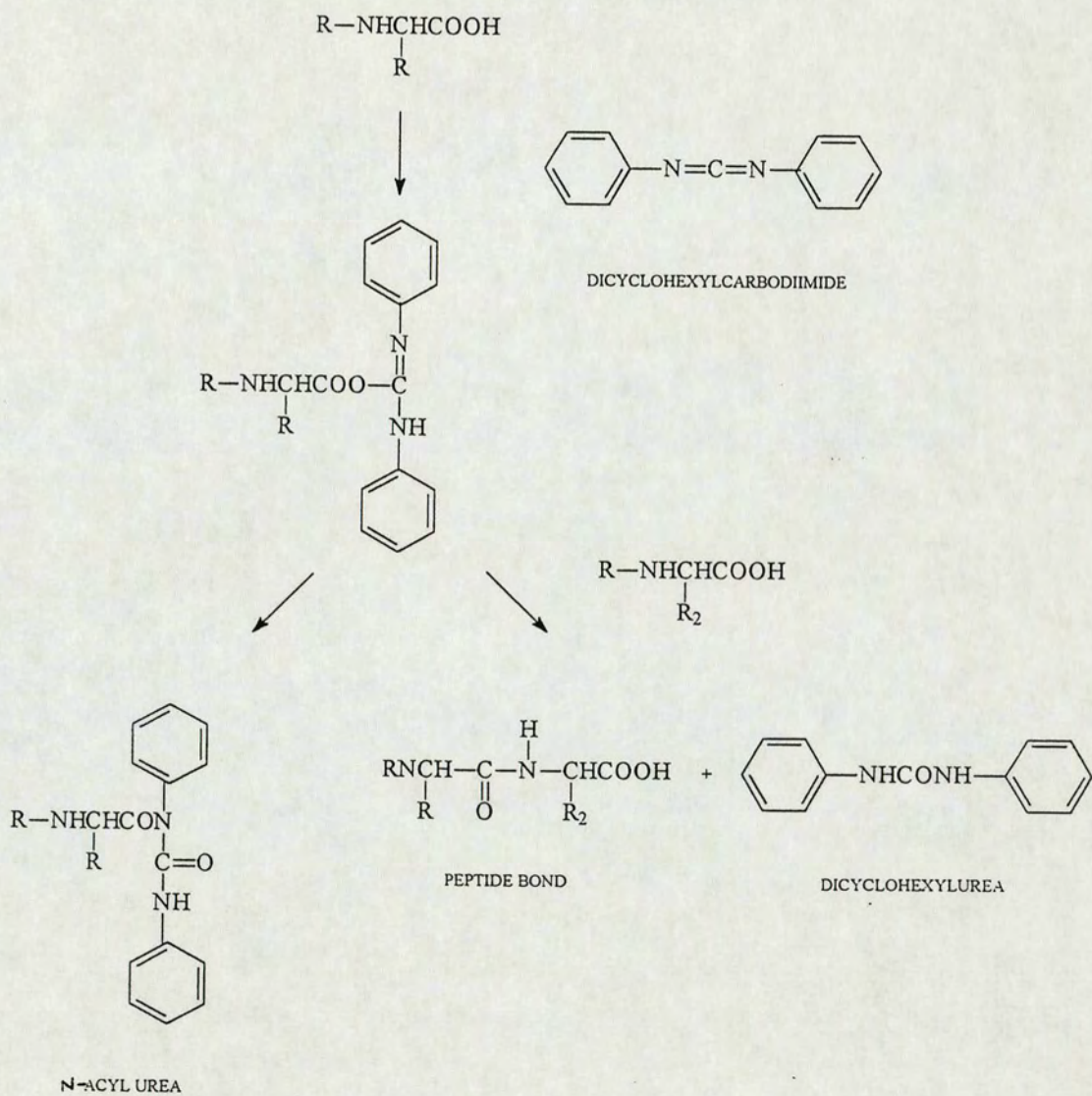


FIGURE 2.4 Synthesis of peptide bonds with carbodiimide (side reactions lead to the less reactive N-acylurea)

poor coupling efficiencies. In addition, the coupling procedures are normally carried out at pH 4-5 pH's which may adversely effect the carrier protein. Problems associated with the use of carbodiimide can be overcome by coupling in the presence of a nucleophile which is able to react rapidly with the O-acylurea before any side reactions can occur to form an acylating agent of lower potency. The resultant carboxylic acid derivative is still reactive with respect to aminolysis but is more discriminating and does not lead to racemisation and other side reactions. Several alternatives have been investigated and the use N-hydroxysuccinimide to generate an active ester (Anderson et al 1963) became popular in the 1970's. Esters of N-hydroxysuccinimide are not only more resistant to hydrolysis than O-acylureas but also allow coupling reactions to be carried out at a physiological pH.

B) CONJUGATION WITH N-HYDROXYSUCCINIMIDE ESTERS

Primary amines are the principal targets of NHS-esters. The amines at the protein N-terminus are seldom available for hapten conjugation and therefore the coupling reactions with amino acid side chains are very important. Cautrecasus and Parikh (1972) compared the reactivity of NHS-esters towards the side chains of various N-acetylated amino acids and free alanine which contains a primary amine. Only lysine is able to react significantly with the NHS-esters through its epsilon amino group. The cysteine thiol group shows some reactivity with the NHS-esters, due to the nucleophilic character of the sulphhydryl group, but the reaction is more efficient with a primary amine.

Hydrolysis of the NHS-ester is a major competing reaction of the NHS-ester acylation reaction. Hydrolysis is a more significant side reaction in dilute solutions. The rate of hydrolysis increases with pH, the half-life of 4-5 hours at pH 7, 0°C decreases to 1 hour at pH 8, 25°C. The rate of hydrolysis of NHS-esters is decreased in dry organic solvents (Carlsson et al. 1978).

Water insoluble and soluble NHS-esters exist. The water soluble forms are characterised by the presence of a sulfonate group on the N-hydroxysuccinimide ring (Figure 2.5a). N-hydroxysulfosuccinimide esters are hydrophilic active esters which hydrolyse slowly compared with their rates of reaction with primary amines (Staros et al, 1986). Staros et al demonstrated that water soluble carbodiimide mediated couplings could be enhanced using N-hydroxysulfosuccinimide, presumably by the formation of the HOSu(SO₃) active ester in situ (Figure 2.5b).

Recent progress in the preparation of homogenous hapten protein conjugates mainly results from the use of heterobifunctional cross-linkers. Fujiewara et al (1990) investigated N-(aminobenzoyloxy) succinimide as a heterobifunctional agent for the preparation hapten-protein conjugates (Figure 2.6). The succinimidyl ester reacts immediately with the amino group and an aminobenzoyloxy group is incorporated into the hapten. The aminobenzoyloxy group is activated by diazotization to a diazobenzoyl group which can couple with the tyrosine and histidine residues of the carrier protein. The reactions are easy to perform, the reagents being added sequentially to a single tube with a change in pH. Acylation of the hapten is time dependent, the longer the incubation the greater the

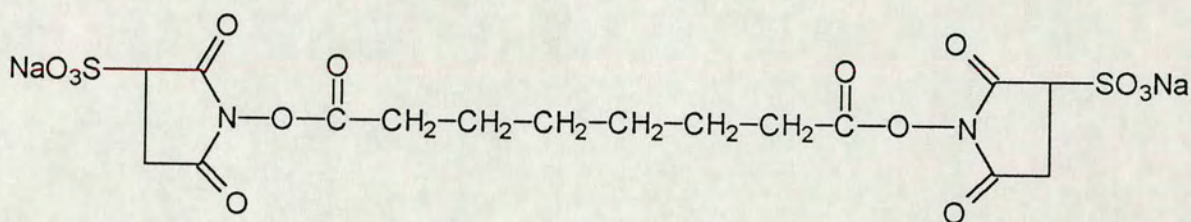


Figure 2.5a The water soluble NHS-ester
bis(sulfosuccinimidyl)suberate (BS^3)

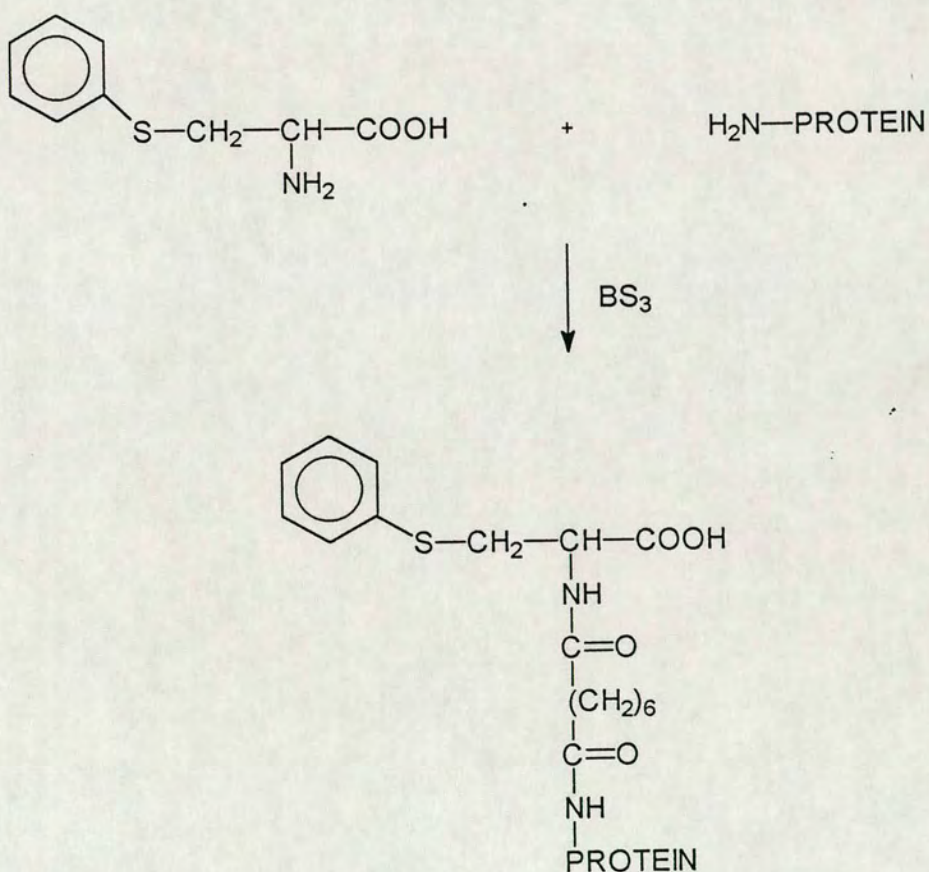


Figure 2.5b Coupling S-PMA to protein with the NHS-ester BS^3 .

incorporation. In addition self-coupling between the aromatic amino group and the succinimidyl ester does not occur during the first acylation at near neutral pH. The azo linkage formed during hapten-protein coupling gives a bright orange-yellow colour. Fujiwara investigated the use of ortho, meta and para-isomers of N-(aminobenzoyloxy)succinimide and found the best coupling efficiencies were achieved with the meta isomer.

2.5 CONJUGATE CHARACTERISATION

Before characterizing the conjugate, non-covalently bound hapten must be removed from the protein carrier. This can be achieved by dialysis or gel filtration. Hapten incorporation can be determined directly by the incorporation of a radioactive tracer into the conjugation procedure. Alternatively if the hapten contains a chromophore which allows it to be differentiated from the protein, the degree of hapten incorporation can be calculated from spectroscopic measurements. If there is overlap in the two spectra, hapten incorporation can be determined from difference spectroscopy.

Sanger (1949) measured hapten incorporation by determining the number of free amino groups on the protein before and after coupling. Substituted and unsubstituted proteins were reacted with dinitrofluorobenzene and the number of dinitrophenyl lysines were determined by spectroscopy after their extraction from an acid hydrolysate. Habeeb (1966) determined the number of free amino groups in protein by reaction with trinitrobenzenesulfonic acid. Primary amines react with TNBS to give trinitrophenyl derivatives. The trinitrophenyl compounds are bright yellow with an absorbance

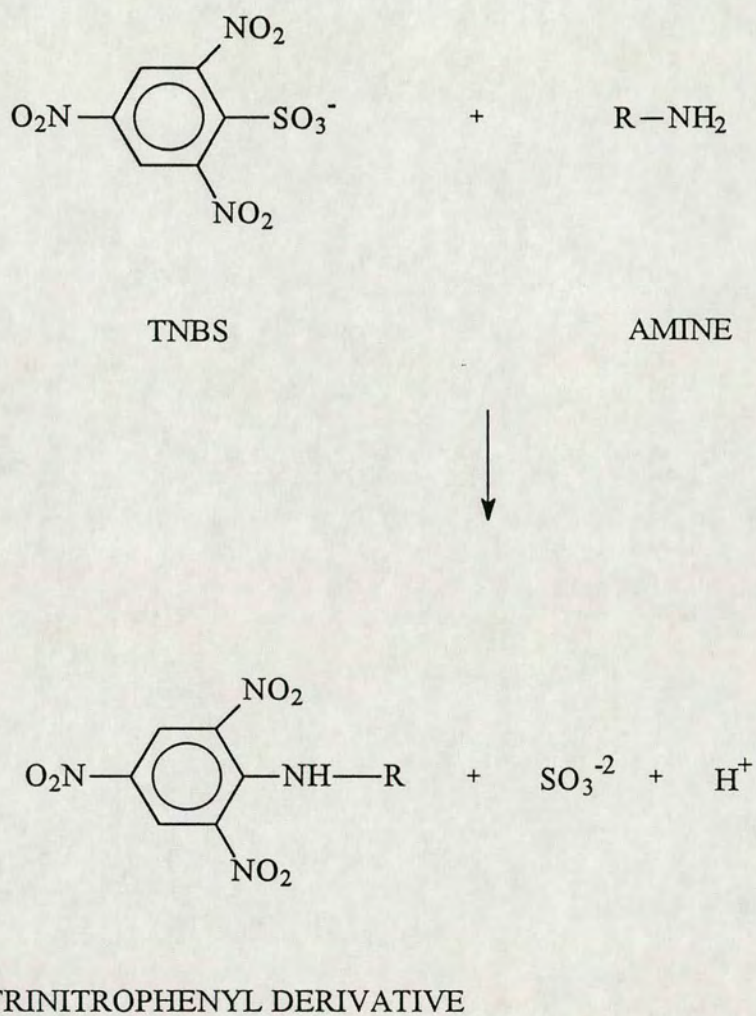


Figure 2.7 The reaction of TNBS with a primary amine gives a yellow trinitrophenyl derivative

maximum at 335nm. Trinitrobenzenesulfonic acid reacts under mild conditions. At pH 8.5-9. Complete reaction with lysine occurs, and there is a linear relationship between absorbance and protein concentration (upto 1mg/ml).

Protein concentrations can be calculated from spectrophotometric readings at 280nm. At which wavelength the absorbance is due primarily to the phenyl groups of the aromatic amino acids. Many of the alternative methods available, (eg. ninhydrin, trinitrobenzenesulfonic acid) depend upon the reaction of the reagent with protein amino groups and are therefore unsuitable for the determination of hapten protein conjugates where the hapten is coupled through this functional group.

When a protein solution is treated with Cu^{2+} in a moderately alkaline solution, a coloured chelate of unknown composition is formed between the Cu^{2+} and carbonyl and imine groups of the peptide bond. The reaction of protein is similar to the reaction of the organic compound biuret and is referred to as the biuret reaction. Cu^{2+} is linked by co-ordinate bonds to 4-6 nearby peptide linkages. There must be at least 2 peptide bonds in the peptide for the reaction to occur. The colour intensity is proportional to the number of peptide bonds available and the biuret reaction forms the basis of several simple colorimetric methods for protein determination.

Lowry et al (1951) studied the use of folin-phenol to enhance the sensitivity of the biuret reaction. This reagent appears to have several advantages - it is 10-20 times more sensitive than UV



absorption at 280nm, is several times more sensitive than ninhydrin reagent and 100 times more sensitive than the biuret reaction. Two distinct steps lead to the final colour: 1) the reaction with copper in alkaline conditions; 2) the reduction of folins reagent at pH 10. At this pH the reagent is only reactive for a short time which makes this an exacting technique with respect to reagent addition and mixing.

The sodium salt of bicinchoninic acid is a water soluble compound which is highly specific and sensitive for Cu^{1+} , forming a stable coloured complex. Smith et al (1985) replaced Folins reagent with bicinchoninic acid in the method of Lowry to give a more simple technique. In addition, Smith found the bicinchoninic acid method was more sensitive than the Lowry assay, was more tolerant towards interfering substances, and possessed greater working reagent stability.

In general, methods for determining protein concentration using dyes are insensitive. Bradford et al (1975) described a sensitive method (4x Lowry assay) for determining protein concentration using the dye Coomassie Brilliant Blue. The binding of Coomassie Brilliant Blue to protein causes a shift in the absorption maximum of the dye from 465 to 595nm. The increase at 595nm is measured. Bradford's assay is rapid (2 min), reproduceable and relatively free from interferences. Coomassie Brilliant Blue was used to determine protein concentrations and the results were compared to the values obtained with bicinchoninic acid.

2.6 IN-VIVO IMMUNISATION

At present mice are the animals of choice for immunisation, and a number of different species are available. There is considerable variation between species in the response to antigen and the best responders vary according to the antigen. Balb/c mice however are the simplest and preferred choice for immunisation. If the spleen cells are not of Balb/c origin and the hybridomas are to be grown as tumours in mice to produce antibody, the hybridomas will only grow in a Balb/c F1 hybrid. Several mouse myelomas are available. Although these possess different properties they are all of Balb/c origin.

Immunisation causes the B cells to divide and differentiate. Expanding the number of relevant spleen cells increases the chance of obtaining the desired hybrid. In addition, activated B cells appear to fuse preferentially.

Soluble proteins are often weakly immunogenic. Their immunogenicity can be increased by the use of adjuvants (Oi et al 1978). The usual practice is to emulsify aqueous antigen with Complete Freund's Adjuvant (containing heat killed *M. tuberculosis* in an oily suspension) for the primary immunisation, and with Incomplete Freund's Adjuvant for subsequent boosts. The stable water in oil emulsion probably acts as a depot allowing a slow and steady release of antigen from the site of injection. The adjuvant may also stimulate local inflammatory reactions (Goding 1986).

Various immunisation protocols have been successfully used. In general the initial immunisation is given either subcutaneously or

interperitoneally in Complete Freund's Adjuvant. One or more subsequent boosts in Incomplete Freund's Adjuvant are normally administered by the same route 21 days later. The final boosts in saline are given either interperitoneally or intravenously 3-4 days before a fusion.

Purity of the antigen is usually irrelevant unless the impure material gives a weaker response, or the screening assay does not distinguish between the component of interest and the impurity.

The chances of obtaining a monoclonal antibody with particular properties is directly related to the concentration of corresponding antibody in the serum of the spleen donor. The magnitude of the response reflects the proportion of spleen cells secreting the desired antibody in the total population. The maximum yield of positive hybrids is obtained 3-4 days after the final boost. The maximum antibody titre occurs about 14 days after the second immunisation. The optimum screening time is therefore after the penultimate boost.

S-PMA and 2-HEMA are low molecular weight compounds (MW 239 and 220 respectively). To generate compound specific antibodies to S-PMA mice were immunised with S-PMA and S-phenylcysteine protein conjugates. This chapter describes the preparation of S-PMA protein conjugates using either carbodiimide or N-hydroxysuccinimide chemistry.

The synthesis of 2-HEMA, S-(2-hydroxyethyl)cysteine and 2-HEMA protein conjugates is described. 2-HEMA and S-(2-

hydroxyethyl)cysteine were prepared by reacting the nucleophilic anion of acetylcysteine with ethylene oxide. 2-HEMA protein conjugates were prepared with both homobifunctional (bis(sulfo)succinimidyl suberate) and heterobifunctional (m-aminobenzoyloxysuccinimide) crosslinkers. 2-HEMA appears to a weakly immunogenic and in order to improve antibody induction the role of the carrier protein was investigated. Coupling 2-HEMA to gelatin and homologous proteins was investigated.

To generate class-specific antibodies mice were immunised with acetylcysteine coupled through the sulfhydryl group to the carrier protein. Acetylcysteine was coupled with Iodoacetic acid N-hydroxysuccinimide.

Protein conjugates were fully characterised, hapten-protein ratios were determined either spectrophotometrically or by amino acid analysis. Balb/c mice were immunised. Sera were screened by ELISA and RIA for antibodies which bind free mercapturic acids. The difficulties of generating antibodies to small MW compounds and the results of the screening strategies are discussed.

METHODS AND MATERIALS

MATERIALS

REAGENTS

The following reagents were purchased from Aldrich Chemical Co. Ltd., Gillingham, Dorset. UK.:- N,N-Hydroxysuccinimide and N,N-dicyclohexylcarbodiimide,

The following reagents were purchased from BDH Merck, Merck Ltd., Dagenham, Essex. UK.:- Acetic acid (chromatography grade), acetic anhydride (AnalaR), ammonia (0.888), butanol (GPR), chloroform (AnalaR), citric acid (AnalaR), dimethylformamide (DMF, GPR), dimethylsulfoxide (DMSO, GPR), di-sodium hydrogen phosphate (AnalaR), formic acid (HPLC grade), hydrochloric acid (GPR), Kiesel gel 60, methyl chloride (AnalaR), potassium dihydrogen phosphate (AnalaR), potassium hydroxide (AnalaR), sodium bicarbonate (AnalaR), sodium carbonate (AnalaR), sodium chloride (AnalaR), sodium dihydrogen orthophosphate (AnalaR), sodium dodecyl sulphate (SDS), sodium hydroxide (AnalaR), sodium nitrite (GPR), toluene (GPR) urea (AnalaR) and kieselgel 60 F254 TLC plates.

2,2-azino-di-[3 ethylbenz-thiozoline sulphonic acid] was purchased from Boehringer Mannheim, Lewes, E. Sussex. UK.

Second antibody, peroxidase-conjugated rabbit immunoglobulins to mouse immunoglobulins, was purchased from Dakopatts a/s, High Wycombe, Bucks. UK.

β -Alanine and ethylenediamine were obtained from Fluka Biochemika,

Gillingham, Dorset. UK. Ethyl acetate (AnalaR), charcoal and sodium acetate (GPR) were supplied by Hopkins and Williams Ltd., Chadwell Heath, Essex. UK

S-PMA was obtained from Janssen Chimica, Hyde, Cheshire. UK.

G100-Sephadex was supplied by Pharmacia Ltd., Milton Keynes, Bucks. UK.

Bis(sulfosuccinimidyl)suberate (BS³), bichinchonic acid and coomasie blue protein assay kits were purchased from Pierce Chemicals Ltd., Luton. UK.

Acetonitrile (HPLC grade), methanol (HPLC grade), and tetrahydrofuran (THF) were purchased from Rathburns Ltd., Wakerburn, Scotland. UK.

The following reagents were purchased from Sigma Chemical Co. Ltd., Poole Dorset UK.:- acetylcysteine, m-aminobenzoic acid, bovine serum albumin (catalogue no. A7030), cysteine, cystine, dextran (clinical grade), diaminopentane, p-dimethylaminobenzaldehyde, dithiothreitol, ethylene oxide, N-ethyl-N-(3-dimethylamino-propyl)-carbodiimide (EDC, protein sequencing grade), Ellman's reagent, N-ethylmaleimide, Freund's complete and incomplete adjuvant, gelatin (Type A 60 bloom), glutathione (GSH), human serum albumin (catalogue no. A1887), hydrogen peroxide, iodoacetic acid-N-hydroxysuccinimide, Keyhole Limpet hemocyanin *Megathura Crenulata*, (catalogue no. H2133), mouse serum albumin (catalogue no. A1056), suberic acid di-N-hydroxysuccinimide, trinitrobenzenesulfonic acid (TNBS), Trizma base (reagent grade), Tween-20.

PBS buffer tablets (Oxoid) were from Unipath, Basingstoke. UK.

ANIMALS

Balb/C mice were supplied by Charles River, Margate, Kent. UK.

PLASTICS

Nunc Immunoplates F96 cert and Covalink microtitre plates were purchased from Gibco Ltd., Paisley, Scotland. UK.

BUFFERS

ABTS substrate consisted of 2,2'-azino-di-[3-ethylbenz-thiozoline sulphonic acid] (0.63mg/ml) in citrate phosphate buffer (0.1M, pH 4.3) containing 0.0036% hydrogen peroxide.

0.1M Citrate buffer pH 4.3 consists of 0.1M citric acid and 0.1M disodium hydrogen orthophosphate.

10mM Acetate buffer pH 6.2 consisted of 10mM sodium acetate adjusted to pH 6.2 by the addition of 10mM acetic acid.

0.1M Carbonate buffer pH 8 consisted of 0.1M sodium carbonate and 0.1M sodium bicarbonate.

Coating buffer (pH 9.8) consisted of sodium carbonate (0.015M) and sodium bicarbonate (0.03M).

0.1M Phosphate buffer pH 4.5 consisted of 0.1M sodium dihydrogen orthophosphate adjusted to pH 4.5 by the addition of 0.1M sodium hydrogen orthophosphate.

0.1M Phosphate buffers pH 7.5, 7.6 and 8 consisted of 0.1M disodium hydrogen phosphate adjusted to pH by the addition of 0.1M sodium dihydrogen phosphate.

0.1M Phosphate buffer pH 7.0 containing 3M urea consisted of 0.1M potassium dihydrogen phosphate (390ml), 0.1M sodium hydrogen phosphate (610ml) and 3M Urea.

0.01M Phosphate buffer pH 7.2 containing 0.14M sodium chloride consisted of 0.14M sodium chloride in 0.01M sodium hydrogen phosphate, adjusted to pH 7.2 by the addition of 0.01M potassium dihydrogen phosphate.

0.05M Phosphate buffers pH 7.4 and 8 consisted of 0.05M sodium hydrogen phosphate adjusted to pH 7.4 and pH 8 by the addition of 0.05M potassium dihydrogen phosphate.

Phosphate buffered saline (PBS) pH 7.4 was prepared from tablets (Oxoid), 1 tablet/100ml distilled water.

PBS/Tween consisted of 0.05% w/v Tween-20 in PBS pH 7.4.

Saline/Tween wash solution consisted of 0.9% w/v saline, 0.05% w/v Tween-20 in distilled water.

0.1M Tris/HCl pH 9.0 consisted of 0.1M Trizma base adjusted to pH 9.0 by the addition of 0.1M HCl.

METHODS

S-PHENYL-N-ACETYLCYSTEINE

2.7 CONJUGATION OF S-PHENYL-N-ACETYLCYSTEINE TO BSA AND KLH BY MEANS OF 3-(3-DIMETHYLAMINOPROPYL)CARBODIIMIDE WITH DIAMINOPENTANE AS A SPACER ARM.

S-PMA was coupled to BSA and KLH as follows. BSA (60mg, 10mg/ml) and KLH (60mg, 5mg/ml) in 0.1M phosphate buffer pH 8 was added to an excess of diaminopentane (1.03ml and 70ul respectively) with stirring. EDC (1.66 and 0.115g) was added with stirring to the mixture and the solution was reacted with continuous stirring at room temperature for 2 hours. The solution was dialysed extensively first against distilled water then against 0.1M phosphate buffer pH 8. Diaminopentane in the dialysate was monitored with the colourimetric reagent p-dimethylaminobenzaldehyde (amines condense with p-dimethylaminobenzaldehyde to yield a yellow schiff base).

Briefly, a drop of a saturated solution of p-dimethylaminobenzaldehyde in toluene was dropped onto a filter paper, spotted with dialysate and heated in an oven at 100°C for 4 minutes. After removal of the diaminopentane the protein concentrations were determined from spectrophotometric readings at 270nm.

S-PMA (10mg/ml in 10% v/v methanol water) was reacted stoichiometrically with EDC at 4°C for 30 minutes with stirring. An excess (x100 moles) of activated S-PMA (0.724ml) was added to the protein-diaminopentane spacer solutions (20mg BSA 2.45 mg/ml, 20mg KLH 1.6 mg/ml respectively) with stirring. The mixtures were reacted

overnight at room temperature with continuous stirring then extensively dialysed against distilled water and PBS. Protein concentrations were determined using coomassie blue (2.8.1) and bicinchoninic acid (2.8.2) protein assay reagents. After dialysis the concentration of the BSA and KLH conjugates were 1.07 and 0.81 mg/ml respectively. Similar protein conjugate concentrations were obtained with coomassie blue and bicinchoninic acid.

Hapten incorporation ratios were calculated from absorbance readings at 270nm. Hapten incorporation ratios were determined spectrophotometrically. The average hapten incorporation ratio for BSA was approximately 16:1.

2.8 DETERMINATION OF PROTEIN CONCENTRATION

2.8.1 DETERMINATION OF PROTEIN CONCENTRATIONS WITH COOMASIE BLUE.

Coomassie brilliant blue G-250 protein assay reagent was bought ready for use from Pierce Chemicals. BSA and KLH standards (0.125-2mg/ml) were prepared in distilled water. To 0.1ml of standard, blank and sample respectively was added 5ml of coomassie blue assay reagent. The solutions were mixed and the absorbance read at 595nm. The blank absorbance was subtracted from the standard and sample OD readings. The protein concentration in the samples was determined from the calibration plot protein concentration v absorbance.

2.8.2 DETERMINATION OF PROTEIN CONCENTRATION WITH BICINCHONIC ACID

Protein concentration was determined using a Pierce Chemicals bicinchonic acid assay reagent kit. The assay was carried out according to Pierce's instructions. Briefly, BSA and KLH standards (0.125-20ug/ml) were prepared in distilled water. To 0.1ml of standard, blank and sample was added 1ml of freshly prepared reagent, 2 parts sodium carbonate, sodium bicarbonate and sodium tartrate in 0.2N NaOH (solution C) plus 48 parts 4% bicinchonic acid in water (solution B) plus 50 parts 4% cupric sulfate pentahydrate in water (solution A). The solutions were mixed and reacted in a water bath at 60°C for 60 minutes. After incubating the solutions were allowed to stand for 60 minutes and cool to room temperature. The absorbances were read at 562nm. The blank absorbance was subtracted from standard and sample OD values. The protein concentrations in the samples was determined from the calibration plot of standard concentration v absorbance.

2.9 CONJUGATION OF S-PHENYL-N-ACETYL-CYSTEINE TO BSA, HSA AND KLH BY MEANS OF THE HOMOBIFUNCTIONAL CROSS-LINKER BIS(SULFOSUCCINIMIDYL) SUBERATE (BS³).

S-PMA (225mg) was deacetylated by heating with 6M HCl (20ml) at 90°C for 3 hours. The acetic acid, a product of the acid hydrolysis, was removed by rotary evaporation at 35°C. The reaction was monitored by TLC using the solvent mixture butanol, acetic acid and water 3:1:1, TLC plates were developed with both iodine vapour and ninhydrin. A single spot, which gave a positive reaction with ninhydrin, was observed after TLC of the reaction mixture. The R_f values of S-PMA and S-phenylcysteine were 0.77 and 0.64 respectively.

The loss of the acetyl group was confirmed by NMR. NMR was performed on a GE QE300 operating at 300MHz, referenced to TMA at 0ppm. PMA: shift (ppm) 7.18-7.42 (m, 5H, C₆H₅), 4.52 (dd, J=6.0 and 8.1Hz, 1H, CH), 3.10-3.50 (m, 2H, SCH₂), 1.85-1.90 (s, 3H, CH₃). Phenylcysteine: shift (ppm) 7.18-7.42 (m, 5H, C₆H₅), 4.52 (dd, J=6.0 and 8.1Hz, 1H, CH), 3.10-3.50 (m, 2H, SCH₂). CD₄OD was used as solvent.

The S-phenylcysteine was dissolved in di-sodium hydrogen phosphate (60ml) and the pH of the solution (pH 8.5) was adjusted to pH 8 by the addition of 1M sodium dihydrogen phosphate (12ml). 50mg of BSA, HSA and KLH were dissolved in 0.05M phosphate buffer pH 8.0 (5ml, 5ml and 8.33ml respectively). To each protein solution was added 9mg (3.6ml) of S-phenylcysteine with mixing. The homobifunctional crosslinker BS³ (70mg) was dissolved in distilled water (1ml) and immediately added to the protein solutions (21.35mg, 152.5ul) with rapid mixing. The mixtures were reacted overnight with continuous stirring then dialysed extensively first against distilled water then against PBS. The protein concentration was determined using coomasie blue protein assay reagent and the hapten incorporation ratio was measured spectrophotometrically (270nm). Phenylcysteine was coupled to BSA with an average hapten incorporation of 25:1.

2.10 IMMUNISATION SCHEDULE

Primary immunisations (day 1) and secondary immunisations (day 21) were given subcutaneously. Each mouse received 100ug of hapten in 200ul of a 50:50 v/v hapten adjuvant emulsion. Primary immunisations were prepared in Freund's complete adjuvant, secondary immunisations

were prepared using Freund's incomplete adjuvant. Mice were bled (approx. 200ul) from the retro-orbital plexus on day 30.

Serum was separated from whole blood by centrifugation (2200rpm for 5 min), and stored frozen at -70° C.

2.11 SCREENING MOUSE SERA BY ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

2.11.1 COATING PLATES

A) NUNC MAXISORB

Haptens conjugated to Bovine Serum Albumin (BSA) were used for plate coating. A solution of conjugate in coating buffer (1ug/ml) was added (100ul) to the central 60 wells of a microtitre assay plate. The plate was sealed with a plastic cover and incubated for 3 hours at room temperature. The wells were emptied and washed 5 times with saline/Tween solution, shaken dry and stored in the dry at 4° C. until use.

B) NUNC COVALINK

To a solution (1ml) containing 1.87mg/ml of NHS and 3.12mg/ml of EDC in distilled water was added 39.9ugs of S-PMA (3.99ul of 10mg/ml in DMSO). The mixture was reacted for 30 minutes at room temperature with continuous stirring then diluted 1:100 with cold (4°C) 0.1M carbonate buffer pH 8.6. The dilute activated S-PMA was dispensed (100ul/well) into the 96 wells of a covalink NH₂ microtitre plate. The plate was sealed with a plastic film and maintained at 4°C for 30 minutes. The wells were emptied and washed 4 times with PBS/Tween. Plates were shaken dry and stored as above until

required.

2.11.2 SCREENING ASSAY

Sera were diluted with PBS/Tween to give antibody titration curves in the range 1:10 to 1:25,600. Aliquots (100ul) were transferred to the antigen coated wells of a microtitre plate, which was sealed with a plastic film and incubated overnight at room temperature. The wells were emptied, washed 5 times with saline/Tween and shaken dry. Excess wash solution was removed by aspiration. 100ul of anti-mouse IgG peroxidase conjugated antibody diluted 1:1000 in PBS/Tween was added to each well. The plate was covered and incubated for a further 2 hours at room temperature. The plate was washed again and dried, as above, and 100ul of ABTS substrate added. In the presence of peroxidase this substrate turns from a colourless solution to green. Optical density was measured at 405nm using a microtitre plate reader (spectrophotometer).

The ability of an antibody to recognise free hapten was examined in binding inhibition studies. Sera were diluted in PBS/Tween to give a limiting dilution and transferred to the wells of a conjugate coated plate (50ul/well). The sera are incubated in the presence of either S-PMA (50ul/well of 200ug/ml standard), or standard diluent (50ul/well). The assay was performed as described above.

S-(2-HYDROXYETHYL)-N-ACETYLCYSTEINE

2.12 SYNTHESIS OF S-(2-HYDROXYETHYL)CYSTEINE

Cysteine (1.2g, 0.01mol) was dissolved in distilled water (10ml) and cooled to 0°C using an ice water cooling mixture. Potassium hydroxide (1.1g, 0.02mol) was also dissolved in distilled water (3ml) and cooled to 0°C. The potassium hydroxide solution was slowly added to the cysteine with stirring, maintaining the temperature at 0°C.

A 25ml bottle of ethylene oxide (boiling point 10°C) was cooled to -70°C with dry ice. A calibrated pasteur pipette (14drops = 0.176g, 4mmol. of ethylene oxide) was cooled by pumping with the cold ethylene oxide. Using the cold pipette 6 drops of ethylene oxide was transferred to the stirred cysteine solution. The ethylene oxide bottle was stoppered to prevent moisture from entering. After approximately two minutes a further six drops of ethylene oxide was transferred, this was repeated until 36 drops (0.48g, 0.01mol) of ethylene oxide had been added to the cysteine solution. The solution was reacted at 4°C for 16 hours, The mixture was then acidified with 20% concentrated HCl in water to pH 6 with ice water cooling and the solution rotary evaporated at 30°C until dry.

The reaction was monitored and the fractions checked by TLC, using silica gel 254F plates (0.2mm thick, 20cm x 5cm) and either methanol:chloroform 60:40 containing 0.880 ammonia (5 drops in 20mls), or propanol water 1:1 as solvent. Plates were developed using iodine vapour. The best separation was achieved using

propanol:water as the mobile phase, the Rf value of the product was 0.6 and the Rf of cysteine was 0.35. Using methanol:chloroform (60:40) as the mobile phase, the Rf values were 0.25 and 0.5 respectively.

The S-hydroxyethylcysteine was purified by crystallisation from methanol water 80:20. The S-hydroxyethylcysteine was dissolved in a minimum amount of solvent at room temperature and cooled in a fridge overnight. Crystals were removed by filtration, washed quickly with a small amount of methanol water (95:5) and dried in a desiccator with molecular sieve. The remaining filtrate was rotary evaporated and recrystallised as above. Rotary evaporation and crystallisation produced a white solid. NMR [shift (ppm) 3.88 (dd, J=6.0 and 8.1Hz, 1H, CH), 3.69 (t, J=6.6Hz, 2H, CH₂OH), 3.1 and 2.98 (dAB, J=15.0, 8.1 and 6.0Hz, 2H, SCH₂CH), 2.70 (t, J=6.6Hz, 2H, SCH₂)] (solvent D₂O) and MS analysis (+ve CI: M+H 166 (40%), 149(100%) confirm product was S-(2-hydroxyethyl)cysteine. Optical rotation measurements with L-valine and S-(2-hydroxyethyl)cysteine indicate racemisation in the presence of KOH did not occur.

2.13 SYNTHESIS OF S-(2-HYDROXYETHYL)-N-ACETYLCYSTEINE

The preparation of this compound was as for S-hydroxyethyl cysteine, using 1.6g of N-acetylcysteine.

Hydroxyethylation of acetylcysteine gave a single product when the reaction mixture was monitored by TLC. TLC was performed using silica gel 254F plates (0.2 mm thick, 5cm x 20cm) and methanol:chloroform solvent (60:40 or 40:60) containing 0.880

ammonia (5 drops in 20ml). Plates were developed with iodine vapour. The Rf of acetylcysteine was 0.60 and 0.30 and the Rf of S-(2-hydroxyethyl)-N-acetyl cysteine was 0.40 and 0.25 respectively.

Rotary evaporation of the solvent, after HPLC purification, left a clear, oily product. NMR [shift (ppm) 8.42 (s, 1H, NH), 4.45 (dd, J=6.0 and 8.1Hz, 1H, CH), 3.68 (t, J=6.6Hz, 2H, CH₂OH), 3.08 and 2.88 (dAB, J=15.0, 8.1 and 6.0 Hz, 2H, SCH₂CH), 2.72 (t, J=6.6Hz, 2H, SCH₂), 2.02 (s, 3H, CH₃)](D₂O solvent) and MS analysis (+ve CI: M+H 207 (67%), 190 (45%), 166 (15%), 162 (59%), 130 (100%)) confirmed this was S-hydroxyethyl-N-acetyl cysteine (Van Bladeren et al 1980).

The sample was purified using HPLC with the following conditions:

Column:	Spherisorb ODS-2, 5 micron, 25cm x 20mm ID
Detector:	UV, wavelength 210nm; Mass Evaporative Detector 65°C 30psi N ₂ nebuliser flow.
Mobile phase:	10% acetonitrile in water containing 0.03% formic acid
Flow rate:	12ml/min

After HPLC solvent was removed from the S-hydroxyethyl-N-acetylcysteine by rotary evaporation with a water bath at 30°C, traces of formic acid were removed by adding small amounts of water and re-rotary evaporating. Characterisation was as above.

2.14 CONJUGATION OF S-(2-HYDROXYETHYL)CYSTEINE TO PROTEINS

2.14.1 CONJUGATION WITH THE HETEROBIFUNCTIONAL CROSS-LINKING AGENT N-(AMINOBENZOYLOXY)SUCCINIMIDE

A modification of the procedure used by Fujiwara et al (1990) was used to prepare the heterobifunctional crosslinking agent N-

(aminobenzoyloxy)succinimide and hapten-protein conjugates.

A) PREPARATION OF N-(AMINOBENZOYLOXY)SUCCINIMIDE

N-(aminobenzoyloxy)succinimide (ABS) was synthesized as follows. Meta-aminobenzoic acid (1.37g, 10mmol) and dicyclohexylcarbodiimide (2.47g, 12mmol) were dissolved in distilled tetrahydrofuran (THF) (30ml) and cooled, on ice, to 4° C. N-hydroxysuccinimide (1.15g, 10mmol) was added slowly to the mixture with rapid stirring. The mixture was reacted overnight at 4° C. followed by 2 hours at room temperature with continuous stirring. The precipitate (N,N-dicyclohexylurea) was removed by filtration and the filtrate was concentrated by evaporation under a stream of N₂ gas.

The reaction products were separated by column chromatography. The reaction residue was dissolved in a mobile phase (10ml) of mixed solvent (3:1 by volume methyl chloride and ethyl acetate) and loaded on to a Kiesel gel 60 column (2 x 22cm containing 18g dry gel). The order of elution and the position of the reaction products in the column eluent were determined by TLC using Kiesel gel 60 plates (Merck).

Evaporation of the fractions which appeared to contain only m-ABS gave 79mg of a pale brown solid with a melting point of 152-154° C. Mass spectrometry indicated the solid was a mixture of 2 main components and a number of minor components. One of the main components had a molecular weight of 218, as expected. Additional fragments m/z 120 and m/z 136 were consistent with NH₂.Bz.CO and the free amide NH₂.Bz.CONH₂ respectively. NMR studies confirm the

presence of m-ABS and other reaction products [Shift (ppm) 7.10-7.20 (t, 1H, C4), 6.88-6.92 (d, 1H, C5), 6.82 - 6.88 (s, 1H, C1), 6.72 - 6.78 (d, 1H, C3), 1.98 - 2.12 (q, 4H, C8 and C9)](solvent CD₃CN). Undefined peaks at 1.15-1.60 and 1.70-1.85 are probably due to traces of dicyclohexylurea and N-hydroxysuccinimide.

B) CONJUGATION OF S-(2-HYDROXYETHYL)CYSTEINE TO PROTEIN USING ABS AS A CROSS-LINKING AGENT

Incorporation of the m-ABS into the hapten, activation of the aminobenzoyl group by diazotization and its subsequent azocoupling to Bovine serum albumin (BSA) and Keyhole Limpet hemocyanin (KLH) Megathura Crenulata was carried out as follows.

To 1ml of hydroxyethylcysteine (10mg/ml) in acetate buffer (10mM pH 6.2) was added m-ABS (4.12mg, 17.52 μ mol) in 206 μ l of THF. The mixture was reacted at room temperature for 3 hours with continuous stirring, then acidified by the addition of 57.9 μ l of 1M HCl. Diazotization was carried out by the addition of sodium nitrite (3.68mg, 52.57 μ mol in 36.8 μ l of distilled water), and reaction for 15 minutes at 4^o C. with stirring. Activated hapten was added (555.5 μ l) to either BSA (5ml of 1.075mg/ml) or KLH (5ml of 0.85mg/ml) dissolved in Tris/HCl (0.1M, pH 9.0) and reacted for 2 hours at room temperature with continuous stirring. The mixture was then applied to a column of Sephadex G-100 (approx. 2 x 20cm containing 3.6g of dry gel, and a void volume determined with methylene blue) and eluted with phosphate buffer (0.1M, pH 7.0) containing 3M Urea.

The protein-ABS-hydroxyethylcysteine conjugate eluted in the void

volume of the column (azo-coupling S-(2-hydroxyethyl)cysteine to either BSA or KLH gave a bright orange/yellow conjugate). 1ml fractions were collected and absorbance readings were monitored at 280nm. Fractions with the highest protein concentrations were pooled). Protein recoveries (pooled fractions) of 59% and 61% were obtained for the BSA (0.79mg/ml) and KLH (0.86mg/ml) conjugates respectively. Spectrophotometric analysis demonstrated the qualitative binding of hapten to protein, but it was not possible to calculate spectrophotometrically a quantitative hapten-protein incorporation ratio.

The hapten-protein ratio was determined by amino acid analysis (2.15.2). Pooled conjugates were used to immunize Balb/c mice and for coating ELISA assay plates (2.18).

2.14.2 PREPARATION OF S-(2-HYDROXYETHYL)CYSTEINE - GELATIN CONJUGATES

The preparation of acetylated gelatin, the coupling of the spacer arm B-alanine to the acetylated gelatin and the subsequent conjugation of hapten was based on the method of Marini et al (1989).

A) PREPARATION OF ACETYLATED GELATIN

Acetylated gelatin (Ac-Gel) was prepared as follows. Gelatin was dissolved at 10mg/ml in 6ml of 0.1M phosphate buffer, pH 4.5 (coupling buffer), with gentle heating and stirring. Acetic anhydride (1.2ml) was added slowly, dropwise, with stirring to the gelatin solution. The mixture was reacted for 24 hours with continuous stirring at room temperature then dialyzed extensively

first against distilled water, then against coupling buffer.

After dialysis the Ac-Gel was diluted to 1mg/ml in coupling buffer.

Ac-Gel was stored at -70° C. for future use.

B) CONJUGATION OF CHEMICAL SPACER TO ACETYLATED GELATIN

The spacer arm B-alanine was conjugated to Ac-Gel as follows.

B-alanine (6mg) was added to Ac-Gel solution (30ml, 1mg/ml in coupling buffer). The pH of the clarified solution was adjusted to 4.5 with 1M HCl. N-ethyl-N-(3 dimethylaminopropyl)-carbodiimide ([EDC] 1.5g) was added in 4 equal aliquots, with stirring, while maintaining the pH at 4.5 with HCl. The mixture was reacted for 20 hours with continuous stirring at room temperature and dialyzed extensively first against distilled water, then against coupling buffer. Solutions (0.9mg/ml) B-alanine-Ac-Gel were stored at -70° C. until use

C) CONJUGATION OF S-HYDROXYETHYLCYSTEINE TO SPACER MODIFIED GELATIN

Hydroxyethylcysteine (4.81mg) was dissolved in 5ml of coupling buffer containing B-alanine-Ac-Gel. The pH of the mixture was maintained at 4.5 with HCl. EDC (250mg) was added in 4 equal aliquots as described previously. The mixture was reacted for 24 hours with continuous stirring at room temperature then dialyzed extensively first against distilled water, then against PBS. The concentration of and gelatin-B-alanine-S-(2-hydroxyethyl)cysteine was 0.69mg/ml after dialysis.

2.14.3 CONJUGATION OF S-(2-HYDROXYETHYL)CYSTEINE WITH DI-N-HYDROXYSUCCINIMIDE SUBERIC ACID

A) SYNTHESIS OF N-SUBERYL-S-HYDROXYETHYLCYSTEINE HYDROXYSUCCINIMIDE ESTER (Figure 2.8)

S-hydroxyethylcysteine (165mg) was dissolved in 25ml of DMSO containing 1 to 5% water. Suberic acid di-N-hydroxysuccinimide ester (0.37g) was also dissolved in DMSO containing 1-5% water (5ml). Aliquots (1ml) of S-hydroxyethylcysteine were added every 15 minutes with stirring to the suberic acid di-N-hydroxysuccinimide and the mixture was reacted at room temperature with continuous stirring. The reaction was monitored by HPLC with the following conditions:

Column: Spherisorb ODS-2 5 micron 10cm x 4.6mm ID
Wavelength: 210nm
Flow rate: 1.5ml per minute
Gradient: 10% acetonitrile in water containing 0.03% formic acid to 30% acetonitrile in water containing 0.03% formic acid over 10 minutes, then to 100% acetonitrile over 5 minutes. Hold at 100% acetonitrile. Equilibrate with starting solvent for 10 minutes before injecting the next sample.

S-hydroxyethylcysteine was added (110-123mg) until the amount of mono-substituted product appeared optimal. The monosubstituted product was purified from the disubstituted product and the unreacted suberic acid di-N-hydroxysuccinimide ester by HPLC using the following conditions:

Column: Spherisorb ODS-2 5 micron 25cm x 20mm ID
Detector: ACS Evaporative Mass Detector
Flow rate: 12ml per minute
Gradient: As for analytical runs

The retention times of the S-hydroxyethylcysteine, disubstituted product, monosubstituted product and di-N-hydroxysuccinimide suberic

acid were 2, 10, 12 and 14 minutes respectively (figure 2.9).

Solvent was removed from the collected fractions by rotary evaporation. Traces of formic acid were removed by re-evaporation with water. The structure of the N-suberyl-S-hydroxyethyl cysteine hydroxysuccinimide was confirmed by NMR [shift (ppm) 7.10-7.20 (d, 1H, CONH), 4.50-4.60 (q, 1H, CH), 3.60-3.68 (t, 2H, CH₂OH), 2.80-3.05 (m, 2H, SCH₂), 2.75 (s, 4H, ring), 2.62-2.70 (t, 2H, SCH₂), 2.52-2.62 (t, 2H, OCOCH₂), 2.15-2.25 (t, 2H, NCOCH₂), 1.50-1.70 (m, 4H, CH₂, chain), 1.25-1.45 (m, 4H, CH₂, chain)](CD₃CN solvent).

B) COUPLING N-SUBERYL-S-HYDROXYETHYLCYSTEINE HYDROXYSUCCINIMIDE ESTER TO BSA, KLH AND GELATIN

CONJUGATION 1 (KLH, BSA AND GELATIN)

N-Suberyl-S-hydroxyethylcysteine hydroxysuccinimide ester was conjugated to BSA, KLH and acetylated gelatin incorporating an ethylenediamine spacer arm. The preparation of the acetylated gelatin is described above. Ethylenediamine was conjugated to acetylated gelatin as for B-alanine (described above) substituting B-alanine with 3.6ml of ethylenediamine.

Coupling was carried as follows. 64mg of BSA (51.9mg/ml) and KLH (30mg/ml) in 0.1M phosphate buffer pH 7.5, and 2ml of ethylenediamine spacer acetylated gelatin (0.55mg/ml) in 0.1M phosphate buffer pH 4.5 were cooled on ice. To each solution was added 21.6mg of N-suberyl-S-hydroxyethylcysteine hydroxysuccinimide ester in DMF (65mg/ml) dropwise with rapid stirring on ice. The mixture was allowed to react for 1.5 hours at room temperature with

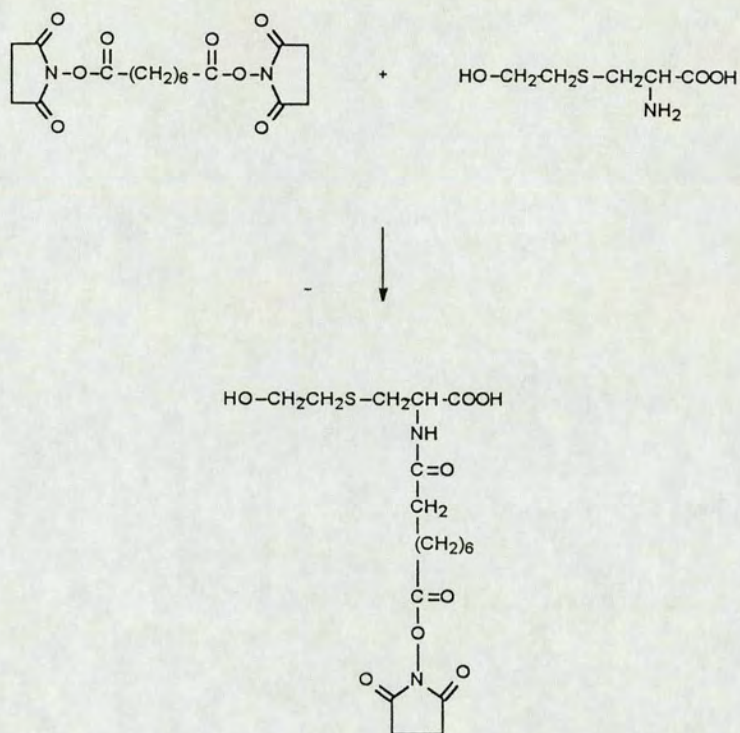


Figure 2.8 Synthesis of N-suberyl-S-hydroxyethylcysteine hydroxysuccinimide ester

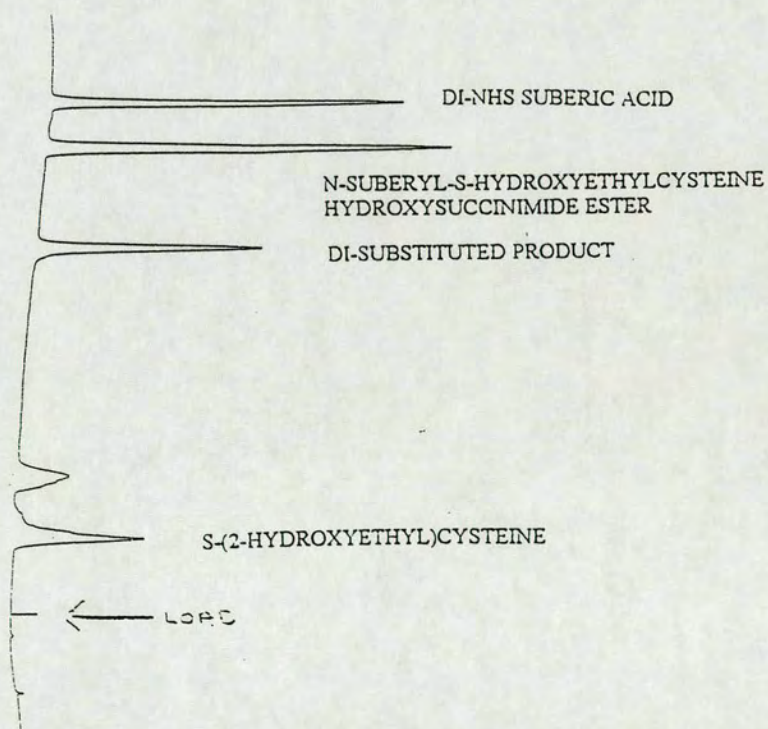


Figure 2.9 Chromatogram showing the separation of N-suberyl-S-hydroxyethylcysteine from other reaction products.

continuous, gentle stirring and was then extensively dialysed against PBS. Protein concentrations were determined from OD readings at 280nm. After coupling, the concentration of the BSA, KLH and gelatin conjugates was 24.5, 18.6 and 0.18mg/ml respectively (recoveries of 74%, 76% and 46%). The hapten protein incorporation ratios were determined using TNBS and by amino analysis (2.15).

CONJUGATION 2 (MSA AND BSA)

Solutions (20mg/ml) of MSA and BSA were prepared in 0.1M phosphate buffer pH 7.6. N-suberyl-S-hydroxyethylcysteine hydroxysuccinimide ester was dissolved in methanol (100mg/ml) and immediately added (61.4ul and 307ul) to the MSA and BSA solutions, 1ml and 5ml, with stirring. The solutions were reacted at room temperature with continuous stirring for 3 hours. To the MSA conjugate was added 1ml of phosphate buffer and both solutions were extensively dialysed, first against water then against PBS. Protein concentrations were determined from OD readings at 280nm. After conjugation the concentration of the BSA and MSA conjugates was 14.3 and 8mg/ml respectively. The incorporation ratios are given below (2.15).

2.15 DETERMINATION OF HAPTEN INCORPORATION RATIOS

2.15.1 DETERMINATION OF THE FREE AMINO GROUPS USING TRINITROBENZENESULFONIC ACID

The number of free amino groups in proteins can be determined using 2,4,6-trinitrobenzenesulfonic acid (TNBS) (Habeeb, 1966). Using the method of Habeeb the number of free amino groups in BSA, KLH and spacer modified gelatin was determined before and after conjugation.

Briefly, to 1ml of protein solution, or water blank, was added 1ml of 4% NaHCO_3 pH 8.5 (adjusted by the addition of HCl) and 1ml of 0.1% TNBS. The solution was mixed and reacted for 2 hours at 40°C . 1ml of 10% sodium lauryl sulfate (SDS) was added to solubilize the protein and prevents its precipitation on addition of 0.5ml of 1M HCl. The absorbance of the solution was read at 335nm.

A linear increase in absorbance was obtained when BSA, KLH and gelatin (0-1mg/ml) were reacted with TNBS. Assuming the molar extinction coefficient of one free amino group is 0.995×10^4 , then 1 mole of BSA contains 51 moles of free amino groups.

In coupling 1 it was estimated that 49% of the free lysines in BSA were conjugated and that the hapten incorporation ratio was 25 moles of 2-HEMA to 1 mole of BSA. Similar percentage conjugations, 50% and 44%, were achieved with the KLH and gelatin conjugates.

In coupling 2 57% of the BSA lysines were conjugated and the incorporation was 29:1. For MSA, after reaction with TNBS, it was calculated that 1 mole MSA contains 43 moles of free amino functions (assuming the MW is the same as BSA, 66000). Reacting the MSA-2-HEMA with TNBS revealed that 45% of the available amino groups were conjugated, and the hapten incorporation ratio was calculated as 19 moles of hapten to 1 mole of MSA.

2.15.2 AMINO ACID ANALYSIS

BSA, KLH and spacer modified gelatin can be degraded to their constituent amino acids by acid hydrolysis. Acid hydrolysis was

performed with 5.7M HCl (constant boiling fraction) in a sealed tube containing N_2 at $110^{\circ}C$ for 90 hours. After hydrolysis the acid was removed by lyophilisation and the sample dried using NaOH. Samples, diluted in loading buffer, were injected onto the amino acid analyser - a cation exchange column from which the eluting amino acids were detected with Ninhydrin reagent (fig. 2.10). By comparison of the retention times of the eluting amino acids with those of a standard solution, containing the common amino acids and S-(2-hydroxyethyl)cysteine, protein amino acid composition can be derived. A calibration run was performed before each sample.

One mole of BSA contained 52 moles of lysine, the hapten-BSA conjugation ratio was 14:1. For the KLH conjugate 37% of the lysines were coupled during the reaction with N-suberyl-S-hydroxyethylcysteine suberate. No 2-(hydroxyethyl)cysteine was observed in the gelatin conjugated sample. An additional peak was observed in the conjugated sample when compared to the gelatin sample which may have been cysteine. However, it was difficult to be certain if this peak (retention time of 28.86) was valine or cysteine due to a slight displacement in the retention times of the sample compared to the standard. In addition, a number of amino acids detected in the gelatin conjugate were not detected in gelatin alone.

2.16 CONJUGATION OF N-ACETYLCYSTEINE TO MSA AND BSA WITH IODOACETIC ACID N-HYDROXSUCCINIMIDE AS THE CROSSLINKER (Figure 2.11)

MSA (45mg) and BSA (100mg) were dissolved in 0.05M phosphate buffer pH 8 to give two 10mg/ml solutions. To 40mg of MSA and to 90mg of

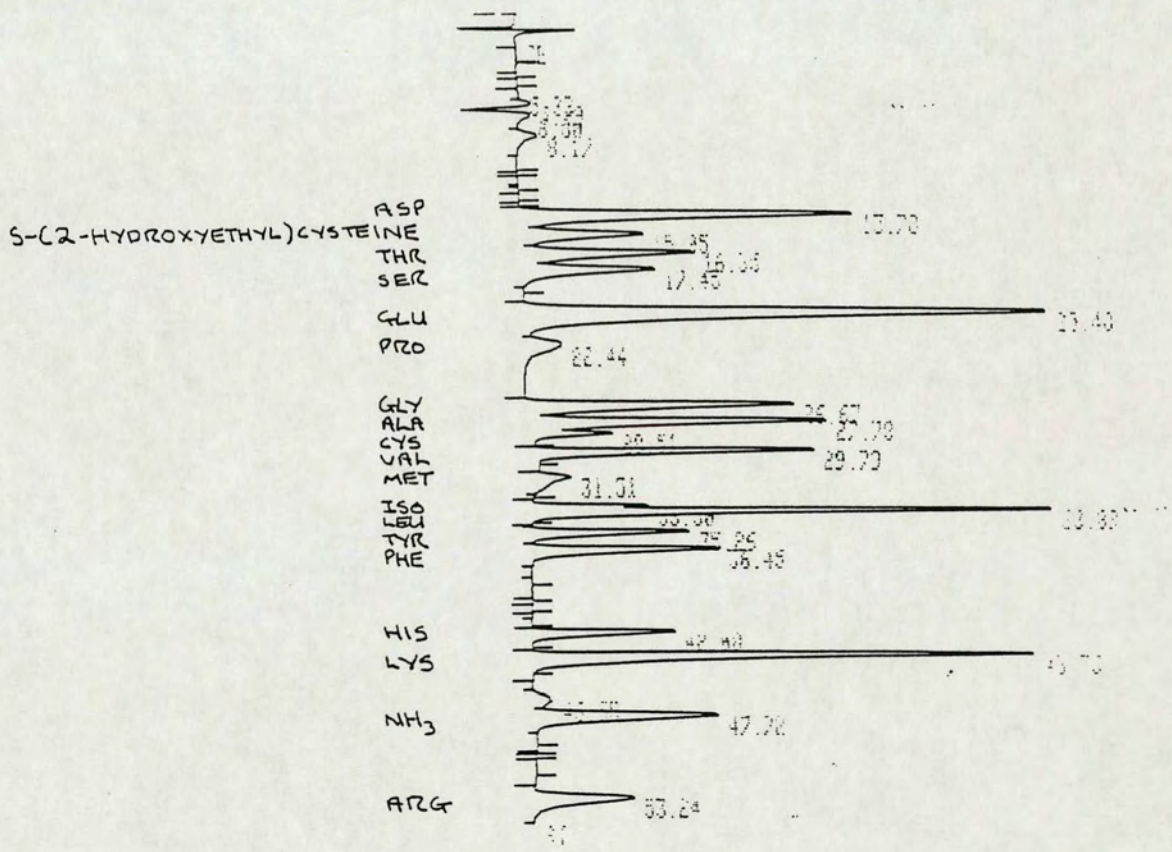


Figure 2.10 Chromatogram showing the separation of S-(2-hydroxyethyl)cysteine and protein amino acids achieved during amino acid analysis.

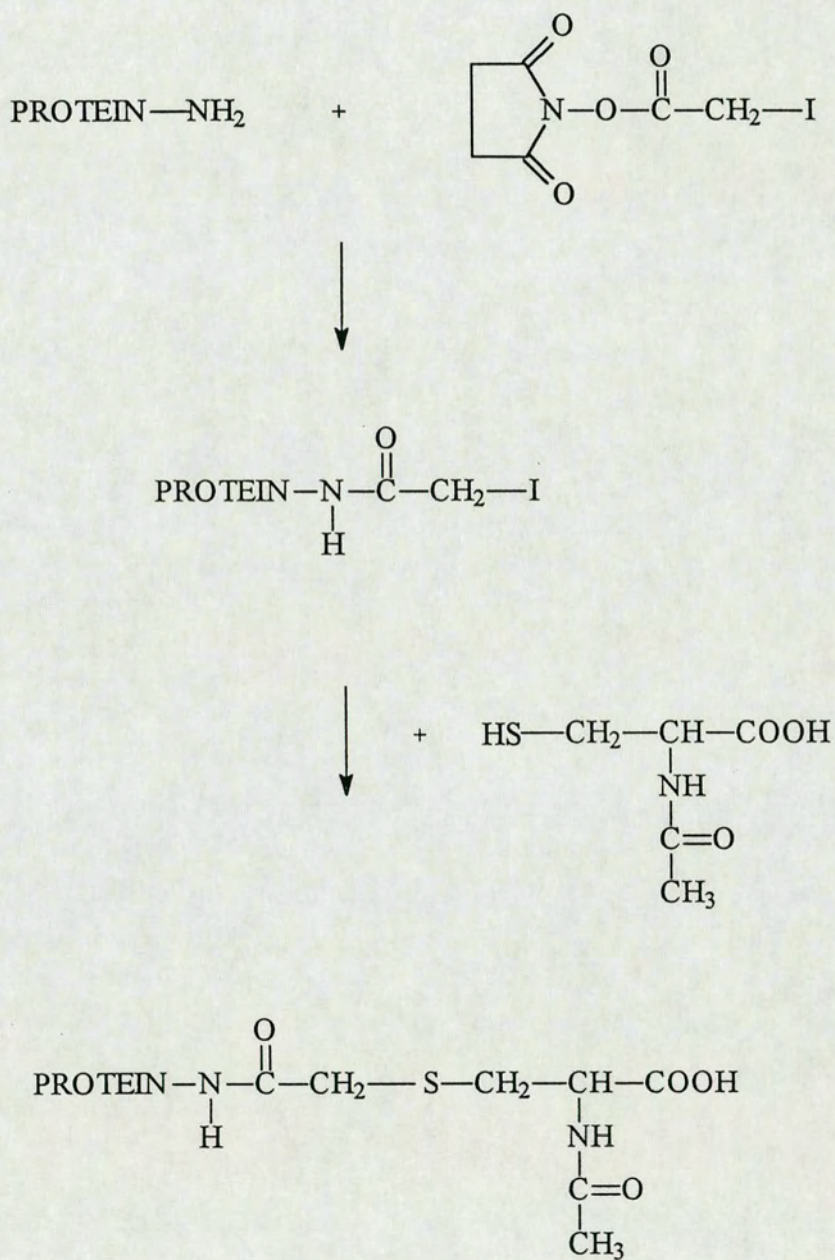


Figure 2.11 Conjugation of N-acetylcysteine to protein with Iodoacetic acid N-hydroxysuccinimide.

BSA was added 26.5 and 60mg of Iodoacetic acid N-hydroxysuccinimide dissolved in DMSO (194 mg/ml). The mixture was reacted overnight at room temperature with continuous stirring, then dialysed extensively against 0.05M phosphate buffer pH 7.25. The reaction was monitored with the colourimetric reagent TNBS and 90% of the available protein amines were conjugated. After dialysis the MSA and BSA spacer concentrations were 10.5 and 10.2 mg/ml respectively.

N-Acetylcysteine was linked through its sulfhydryl group to the halogen activated proteins as follows. To 40mg of activated MSA and 90mg of activated BSA was added 24ul and 55ul respectively of 100mg/ml N-acetylcysteine dissolved in distilled water. The mixtures were reacted overnight at room temperature with continuous stirring, then dialysed extensively against PBS. The reaction of N-acetylcysteine with protein was monitored by measuring the fall in the number of free sulfhydryl groups with Ellman's reagent.

Ellman's reagent (5,5'-dithio-bis-(2-nitrobenzoic acid) was dissolved (4mg/ml) in 0.1M disodium hydrogen phosphate and adjusted to pH 8 by the addition of 0.1M sodium dihydrogen phosphate. To 100ul of sample was added 100ul of Ellman's reagent and 5ml of 0.1M phosphate buffer pH 8. The solution was mixed and reacted at room temperature for 20 minutes. The absorbance was read at 412nm - at this wavelength the extinction coefficient (E_{412}) equals $1.36 \times 10^4 \text{ cm}^{-1}\text{M}^{-1}$. The average hapten incorporation ratios were 25:1, determined from the number of free sulfhydryl measured before and after coupling. The protein concentration after dialysis was 10mg/ml.

2.17 SYNTHESIS OF RADIOLABELLED S-(2-HYDROXYETHYL)-N-ACETYL-CYSTEINE

Carbon-14 labelled cystine (302 mCi/mmol) was obtained from NEN radiochemicals. The cystine was labelled on all the carbon atoms. To 100 μ Ci of cystine in 0.5ml 2% ethanol in water (as supplied) was added 10mg of N-hydroxysuccinimide acetate (in 100 μ l of DMF), and 25 μ l of a 1% solution of sodium carbonate in water. The mixture was reacted at room temperature for two hours and monitored by TLC. Samples were spotted onto F254 silica coated plates (0.2mm thick, 5 x 20 cm), and developed using either propanol:water (1:1), butanol:acetic acid:water (3:1:1) or chloroform:methanol (6:4) containing 5 drops of 0.222 ammonia in 20ml of solvent. The components were detected by radioscanning. The best chromatography was achieved using propanol-water as the solvent. The reaction was complete after 2 hours giving a single spot in the N,N-diacetylcystine position.

Dithiothreitol (3mg) was added directly to the reaction and the mixture was heated at 60°C for 5 hours under a nitrogen atmosphere. The mixture was examined by TLC using the above conditions. In addition, a sample of the reaction was co-chromatographed with cold acetylcysteine. After TLC the products were visualised with iodine vapour. The labelled product had the same R_f value as cold acetylcysteine whilst the cold acetylcysteine doped with labelled product coeluted.

Confirmation of the result was obtained by HPLC. The retention time of the radio peak was compared to the retention time of cold acetylcysteine in a sample of cold acetylcysteine spiked with the hot

reaction product. The conditions were as follows:-

Column: spherisorb SAX 5 micron 15cm x 4.6mm ID
Mobile phase: 30% methanol in water containing 0.1% formic acid
Flow rate: 1 ml/minute
Detector 1: UV detector wavelength 220nm
Detector 2: Ramona Raytest detector.

The retention time of the radio peak (in line radiochemical detection) was coincident with cold acetylcysteine (UV detection).

Potassium hydroxide solution (0.1% in water) was added to the reaction mixture until the pH was 14. The mixture was cooled in ice-water and 1ml of ethylene oxide was added. The reaction was monitored by HPLC using the conditions described above. When the reaction was complete the pH was adjusted to 6 by the addition of 30% hydrochloric acid in water. The sample was stored overnight at -30°C (16 hours). When the mixture was thawed and checked by HPLC it was found to contain another component close to the solvent front.

The sample was isolated by HPLC, using the conditions described above, and reinjected to confirm the retention time with cold S-(2-hydroxyethyl)-N-acetylcysteine. The result was checked by radio TLC with confirmation by doping with cold S-(2-hydroxyethyl)-N-acetylcysteine and detection with iodine.

Isolated fractions were radiocounted and freeze dried. Samples of freeze dried material were also counted and the activity of the prepared S-(2-hydroxyethyl)-N-acetylcysteine was calculated. The total activity of the labelled 2-HEMA prepared was 150mCi/mmole

2.18 IMMUNISATION AND SCREENING

Mice were immunised with the 2-HEMA KLH, MSA and gelatin conjugates using the protocol described above (2.10). The sera were screened by ELISA against BSA 2-HEMA conjugates. In addition sera were screened for antibodies by RIA.

2.18.1 RADIOIMMUNOASSAY OF 2-(HYDROXYETHYL)ACETYLCYSTEINE.

Labelled 2-HEMA was diluted in PBS on the day of use so 100ul contained 10 000 dpm. To 100ul of serum diluted 1:10 in PBS and a control blank serum (serum from a mouse immunised with a different conjugate) was added 100ul of labelled 2-HEMA. Totals containing 100ul of label only were also prepared. All the samples were capped and reacted overnight at room temperature.

SEPARATION OF BOUND AND FREE LABEL.

Preparation of 5% dextran coated charcoal:-

Charcoal (5.0g) and dextran (0.5g) was first mixed to a smooth paste with a few drops of PBS and the volume made up to a 100ml by the slow addition of PBS with continuous stirring. The mixture was stirred at 40°C for 90 minutes before use.

Separation:-

To all the samples and the blank was added 100ul of 5% dextran coated charcoal (at 40°C). After incubation at room temperature for 5 minutes, the charcoal was separated by centrifugation (2400rpm for 20 minutes) and the radioactivity in the supernatant counted. The radioactivity in the supernatant is directly proportional to the amount of labelled analyte bound by the antibody.

RESULTS

S-PHENYL-N-ACETYLCYSTEINE

2.19 BINDING STUDIES WITH MOUSE SERA

Mice were bled 14 days after the second immunisation yielding 50-150ul of serum per mouse. Sera were screened for the presence of antibody by ELISA. All the screened sera contained antibody which bound to the conjugate coated plates. The antibody titre (sera concentration giving half the maximum OD) varied between mice immunised with the same conjugate. Mice (x2) immunised with KLH-S-PMA had the following titres :-

MOUSE	PLATE	
	COVALINK	BSA-PMA
1	1:1500	1:2000
2	1:2000	1:3000

When substrate was added at the end of the screening assay, higher optical densities were observed in the wells of the covalink plates. None of the antibodies bound free S-PMA (200ug/ml) in the inhibition studies.

Antibody titres of 1:20 000-1:25 000 were obtained with phenylcysteine conjugates (figure 2.12). Competitive inhibition using 200ug/ml of free S-PMA resulted in a reduction of binding of 3-26% in the ELISA.

IMMUNOGEN	MOUSE	TITRE	%INHIBITION
HSA-PMA	1	1:20 000	8
	2	1:25 000	9
	3	1:20 000	33
KLH-PMA	1	1:25 000	22
	2	1:20 000	3
	3	1:25 000	26

Figure 2.12 Titre and relative affinity of antisera from mice immunised with S-Phenylcysteine conjugates.

S-(2-HYDROXYETHYL)ACETYL-CYSTEINE

2.20 BINDING STUDIES WITH MOUSE SERA.

Sera were screened for the presence of antibodies which recognise 2-HEMA by ELISA. All the sera contained antibody which bound conjugated hapten. The highest titres (1:4000-1:20000) were obtained with the KLH-conjugate immunised mice (Figure 2.13). Titres obtained with 2-HEMA gelatin and MSA conjugate-immunised mice were similar, 1:15-1:150 (Figure 2.13). MSA-acetylcysteine-immunised mice had titres of 1:600-1:2500. The inhibition of colour development (1-57%) showed the bleeds from 18 out of 19 mice contained some antibodies which recognised free 2-HEMA (Figure 2.13 and 2.14). Mice immunised with the gelatin and MSA conjugates gave the largest percentage inhibition values (Figure 2.13 and 2.14).

Mice immunised with MSA-acetylcysteine conjugates were screened against acetylcysteine, 2-HEMA and S-PMA. As the molecular weight of the hapten increased the percentage inhibition in the binding study also increased. The percentage inhibition observed with S-PMA was

also increased. The percentage inhibition observed with S-PMA was 1.6 to 3.8 times greater than that observed with 2-HEMA (Figure 2.14).

Figure 2.13 RESULTS OF 2-HEMA MOUSE SCREENING ASSAYS

IMMUNOGEN	MOUSE	PLATE COATING	TITRE	% INHIBITION
KLH-ABS-HEMA	1	BSA-ABS-HEMA	1:2000	11%
	2		1:4000	4%
	3		1:5000	10%
GEL-BALA-HEMA	1	BSA-ABS-HEMA	1:300	21%
	2		1:200	16%
	3		1:300	5%
KLH-7C-HEMA	1	BSA-7C-HEMA	1:20000	13%
	2		1:9000	3%
	3		1:20000	11%
GEL-7C-HEMA	1	BSA-7C-HEMA	1:150	3%
	2		1:15	57%
	3		1:100	1%
MSA-7C-HEMA	1	BSA-7C-HEMA	1:150	28%
	2		1:100	15%
	3		1:50	0%
	4		1:150	13%

Figure 2.14 RESULTS OF MSA-ACETYLCYSTEINE SCREENING ASSAY

MOUSE	TITRE	% INHIBITION		
		AcCy	2-HEMA	S-PMA
1	1:2500	9	22	84
2	1:600	31	35	81
3	1:600	39	41	69

2.21 RADIOIMMUNOASSAY OF 2-HEMA

Separation of bound and free 2-HEMA was achieved with 5% dextran coated charcoal. Ten percent of the added label was non-specifically bound (NSB). Specific binding was low in all the screened sera. Mouse 2, immunised with gel-7C-HEMA bound 16% of the added label. The other sera bound 10 to 14 percent of the added label.

DISCUSSION

Small molecules (<1000 daltons) are not usually antigenic but can be rendered immunogenic by coupling to large carrier molecules such as KLH and BSA. S-PMA was successfully coupled to KLH and BSA using carbodiimide and diaminopentane as a spacer. Conjugation was demonstrated spectrophotometrically, and the average BSA hapten incorporation ratio was 16:1. The molecular weight of KLH is less precisely known and KLH hapten incorporation ratio was not calculated.

Conjugate concentrations were determined using Coomassie Brilliant Blue and Bicinchonic acid. Comparable results were obtained with the two methods. Lower protein concentrations can be measured with Bicinchonic acid. Both methods are sensitive enough for determining protein conjugate concentrations. Coomassie blue is quick and easy when compared with Bicinchonic acid, and was therefore the method of choice in future work.

Mice were immunised with 100ug of KLH-PMA conjugate. It is important that the immunisation protocol does not expose mice to too much immunogen as this may give rise to low affinity antibodies. High exposures can also cause a failure in the class switching mechanism, and IgG antibodies will not be secreted by the lymphocytes after the second immunisation. The immunisation protocol proved successful as all the sera contained antibodies which bound conjugated hapten.

Sera were screened against BSA-PMA conjugate coated microtitre plates and against S-PMA, covalently coupled to covalink microtitre

plates. S-PMA is covalently coupled through its carboxylic acid to a secondary amino group attached to the end of an 11 carbon spacer. The spacer is hydrophilic and should therefore be fully extended in an aqueous environment to give optimal presentation of hapten to antibody. In addition the method is specific and excludes detection of antibodies which recognise other common epitopes in the coating and immunogen carrier proteins.

Activation of S-PMA (via the carboxylic acid) was performed with high concentrations of NHS and EDC in a short time period (30 minutes) to prevent hydrolysis of the hydroxysuccinimidyl ester formed. It has been reported that coupling high concentrations of activated hapten to covalink microtitre plates gives rise to high assay backgrounds (Sondergard-Anderson et al, 1990). Activated hapten was therefore diluted in carbonate buffer before use. Conjugation of S-PMA to covalink microtitre plates was based on the method of Sondergaard-Anderson (1990).

Titres obtained with the covalink plates were lower than those obtained with the BSA-PMA coated plates. The optical density determined at the end of the screening assay was also higher (approximately 50%) with the covalink plates. These observations can be explained if the coated hapten concentration is greatest in the covalink plates, as a limiting dilution is obtained at a higher antibody concentration. None of the antibodies bound free S-PMA. It was postulated that the antibodies bound either the hapten plus spacer with a greater affinity than hapten alone, or, the COOH group in free S-PMA inhibited antibody binding. To test the latter, S-PMA was deacetylated and coupled to protein through the amino group.

Deacetylation of 2-HEMA using the enzyme acylase 1 has been reported (Gerin and Tardif, 1986). However, efforts to deacetylate S-PMA using acylase 1 in the described conditions were unsuccessful. When acylase was added to S-PMA, enzymic deacetylation started, but soon as phenyl cysteine formed further conversion was inhibited. S-PMA could be deacetylated by hydrolysis using 6M HCl.

Phenylcysteine was successfully coupled to KLH, HSA and BSA using the homobifunctional crosslinker bis(sulfosuccinimidyl)suberate (Figure 2.5a). This cross linker forms an amide bond with the free amino group and the conjugate appears as S-PMA linked through the acetyl's methyl group. The cysteinyl carboxylic acid is left intact (Figure 2.5b). Hapten incorporation could be demonstrated spectrophotometrically for all three conjugates. The hapten to BSA ratio was 25:1.

A large immune response was induced when mice were immunised with KLH and HSA conjugates. Inhibition studies with free S-PMA showed that some of these antibodies recognised free mercapturic acid (Figure 2.12). Mice whose sera showed the greatest inhibition were selected for fusion. The production of a monoclonal cell line which secretes antibody specific for S-PMA is described in chapter three.

A second aim of this project was to generate antibodies which recognise 2-HEMA. This has proved extremely difficult. To generate antibodies which recognise 2-HEMA, the role of the protein carrier was investigated. With a novel, carefully designed and fully characterised hapten-protein conjugate antibodies which recognise conjugated 2-HEMA have been generated. With a judicious screening

strategy it has been shown that these antibodies have a very low affinity for free 2-HEMA, and do not bind hapten free in solution.

2-HEMA standard and S-hydroxyethylcysteine for coupling were synthesized by using the nucleophilic properties of the thiolate anion under alkaline conditions (Figure 2.1a). Acetylcysteine and cysteine were reacted with ethylene oxide in the presence of the base KOH. It was observed that ethylene oxide reacts preferentially with the cysteinyl sulfhydryl moiety and an excess needs to be present before substitution at the amino group of cysteine takes place. It was estimated that recoveries were in excess of 80%. This compares with 82% achieved by Van Bladeren et al (1980) reacting acetylcysteine with hydroxyethyl bromide (Figure 2.2).

For immunisation, 2-hydroxyethylcysteine was initially coupled to KLH using the heterobifunctional cross-linker m-ABS (Figure 2.6). The cross-linker was successfully synthesized by the method of Fujiwara et al (1990), the product indicated by melting point, NMR and mass spectroscopy studies. One advantage of coupling with m-ABS is the colour change when an azo-linkage is formed between an activated hapten and protein. The bright colour indicates a successful conjugation. A hapten incorporation ratio was not determined for the hydroxyethylcysteine m-ABS conjugations. When the same reaction was carried with hydroxyethylglutathione and BSA, however, amino acid analysis showed that 75% of the available tyrosines and histidines were conjugated and the hapten incorporation ratio was 30:1.

A large immune response was induced (1:2000-1:5000) in all the mice

immunised with the KLH conjugate (Figure 2.13). The antibody titre however, is lower than the titres observed in the S-PMA KLH conjugate immunised mice (Figure 2.12). All the sera contained antibodies which recognised free S-HEMA in competitive inhibition studies. The percentage inhibition values (4-11%) were low for all the sera, and therefore only a relatively few hybridoma clones might be expected to secrete antibody specific for 2-HEMA after fusing. This is borne out in fusion results to date (chapter 3). KLH is a highly immunogenic protein whilst 2-HEMA is a small molecule lacking structural features, and the immune response of the mice may be directed towards immunodominant epitopes in the carrier molecule. Gelatin is a well known non-immunogenic carrier protein. Marini et al (1989) conjugated haptens to spacer modified gelatin and induced a strong antigenic response in mice. In an attempt to increase the relative antigenicity of 2-HEMA, hapten was coupled to spacer modified gelatin.

The preparation of spacer modified gelatin and the conjugation of 2-hydroxyethylcysteine was based on the method of Marini. Successful reactions were qualitatively demonstrated by spectrophotometric analysis. The hapten hydroxyethylcysteine absorbed strongly at 190-200nm, although a quantitative estimation of the molar incorporation ratios proved difficult technically. Accurate determinations could not be made for the following reasons:

1. The stacking of absorbance peaks from buffer components.
2. The large dilutions of conjugate needed to bring the absorbance values on to scale.
3. The possible absorbance of any trace impurities at this wavelength.

Marini et al (1989) conjugated haptens containing a chromophore, and were able to determine that 10-12 molecules of hapten were coupled per 100 residues of polypeptide of gelatin.

2-HEMA was also conjugated to protein via a seven carbon spacer arm bis(sulfosuccinimidyl)suberate. This spacer arm should be poorly immunogenic when compared to m-ABS. It would be expected that antibodies which recognise the spacer will bind it with low affinity. Acetylcysteine was coupled via the intermediate N-suberyl-S-hydroxyethylcysteine hydroxysuccinimide (Figure 2.8). Synthesis of this compound allowed easy control of the coupling ratio. In addition, it was possible to easily determine the incorporation ratio by detecting the number of free amines in the carrier protein before and after conjugation. The method should be generally applicable to other mercapturic acids.

N-suberyl-S-hydroxyethylcysteine hydroxysuccinimide was prepared with DMSO containing 1-5% water as the solvent. The 1-5% water is added to prevent excessive di-substitution of the suberic acid di N-hydroxysuccinimide by S-hydroxyethylcysteine. The disubstituted product becomes the predominant and often only product isolable if dry DMSO is used.

Before it was realised that water was effective as a deactivation catalyst other catalysts were added in order to inhibit the reactivity of the amino group of S-hydroxyethylcysteine. Traces of hydrochloric acid prevented the reaction completely. Formic acid gave both mono and disubstituted suberic acid N-hydroxysuccinimide and a large proportion of another unrequired product. N-suberyl-S-

hydroxyethylcysteine hydroxysuccinimide was isolated by HPLC and stored dry at -20°C to prevent hydrolysis of the ester.

N-suberyl-S-hydroxyethylcysteine hydroxysuccinimide was successfully coupled to BSA, KLH gelatin and MSA. The incorporation ratios were determined using TNBS. TNBS reacts with amines to give a yellow trinitrophenyl derivative (Figure 2.7). The reaction of TNBS with BSA, KLH and gelatin is linear over the range 0-1 mg/ml. It was calculated that one mole of BSA contains 52 moles of free lysine. This compares with the ratio obtained by Habeeb (1966) of 60:1. Incorporation ratios were also calculated by amino acid analysis. With amino acid analysis it was calculated that one mole of BSA contains 51 moles of lysine. This value is in reasonable agreement with that obtained with TNBS above. Incorporation ratios calculated by amino acid analysis were lower than those obtained with TNBS. The small differences may be explained by rounding up the ratios in the chemical formula to whole numbers.

Mice immunised with gelatin conjugates all produced antibodies which recognised conjugated hapten. The antibody titres were lower than those observed with the KLH conjugates. Gelatin is non-immunogenic, or poorly immunogenic, and may not be able to stimulate the production of antibodies which recognise common protein epitopes in the coating material.

Sera from mice immunised with the gelatin conjugates had the highest inhibition values. Inhibition values of up to 57% were obtained although in general the percentage inhibition was low (Figure 2.13). Antibodies may be generated which bind a hapten-spacer-protein

complex. Low inhibition values may be explained if the antibodies have a high affinity for the complex and a low affinity for free 2-HEMA. Inhibition studies were carried out with high concentrations of 2-HEMA (100ug/ml). None of the sera were inhibited by non-conjugated BSA.

Sera from mice immunised with the gelatin B-alanine 2-HEMA conjugates were screened against the BSA-ABS-HEMA conjugate. These conjugates differ in carrier protein, spacer and linking chemistry, which involves different protein amino acid residues. Highest inhibition values were obtained when the screening conjugate differed from the immunisation conjugate with respect to carrier protein, spacer and chemical linkage. The importance of screening with conjugates which differ from immunogen in respect to carrier protein and chemical linkage for small haptens has been recently confirmed by Danilova (1994).

In a novel approach to antibody production, MSA was selected as a carrier protein. Using homologous mouse protein all the immune response should be directed towards the hapten. 2-HEMA was coupled to MSA using the intermediate N-suberyl-S-hydroxyethylcysteine hydroxysuccinimide (Figure 2.8). In addition, acetylcysteine was successfully conjugated to MSA using Iodoacetic acid N-hydroxysuccinimide. The N-hydroxysuccinimidyl ester was first reacted with the protein, incorporating an active halogen in to the MSA. Acetylcysteine reacts with the active halogen via its sulfhydryl group, linking the hapten by a short (2 carbon) spacer to the protein (Figure 2.11). Using a short spacer it was hoped the role of bridge in antibody affinity would be lessened without

compromising hapten presentation. In addition antibodies produced to this conjugate should be class-specific - recognising acetylcysteine, they should bind mercapturic acids *per se*.

An immune response was induced in all the mice immunised with the MSA conjugates. Mice immunised with the MSA acetylcysteine conjugates had the greatest titres (1:600-1:2500) when compared to the MSA 2-HEMA conjugates (1:50-150). Reducing the length of the spacer arm does not appear to have affected antibody presentation. In addition, larger percentage values were obtained with sera from mice immunised with the MSA-acetylcysteine conjugates (22-41%) when compared to sera from MSA 2-HEMA immunised mice (0-28%) in the inhibition studies. Using a non or weakly immunogenic carrier molecule with a short protein-hapten bridge it has been possible to focus the immune response and induce antibodies which recognise free 2-HEMA. Inhibition studies were performed with a high concentration of standard (100ug/ml), and from the percentage inhibition it appears that the sera has a low affinity for free hapten.

Sera from mice immunised with the MSA-acetylcysteine conjugate were also screened against acetylcysteine and phenylmercapturic acid (Figure 2.14). As the molecular weight of the inhibitor increased the percentage inhibition in the binding study also increased, confirming that affinity is limited by hapten size. One of the limitations of the ELISA screen is that antibodies which recognise free hapten but bind conjugated hapten with a greater affinity may not be detected in the assay. In order to overcome the limitations of the ELISA a non-competitive RIA was developed.

Hapten iodination, commonly used to prepare radiolabelled antigens, would significantly increase the size of the hapten and may give rise to false positives in the screening assay. It was therefore decided to prepare a C14 labelled hapten with the C14 incorporated into the cysteine molecule.

Using the C14 labelled 2-HEMA a RIA was developed for screening the mouse sera. Dextran coated charcoal was selected to separate free hapten from bound after the reaction of antibody with C14 2-HEMA. In the initial screening assay high levels of non-specific binding (30-35%) were observed. The high NSB appears to be caused by sticking of hapten to Tween 20, since the background can be reduced by the removal of Tween from the assay buffers. After optimisation the non-specific binding was reduced to 10%. Only one sera, from mouse 2 immunised with the gel-7C-HEMA conjugate, showed an appreciable increase over background. This sera also demonstrated the greatest inhibition in the ELISA (57%). In general, antibodies which bound conjugated hapten in the ELISA did not bind to free hapten in the RIA. This appears to confirm that antibodies which recognise derivatised 2-HEMA show low affinity for 2-HEMA. This low affinity is due to the low MW of 2-HEMA.

The relationship between hapten molecular weight and antibody affinity has been reviewed in a recent paper by O. Chappey et al (1994). O.Chappey et al found that for haptens of low molecular weight (MW < 300-325 da) the influence of the linkage arm appears critical, and high affinity antibodies ($10^{-12}M^{-1}$) can only be produced when using a chemical entity resembling an immunogenic conjugate. A MW of approximately 350 appears optimal for maximum

interaction with the antibody binding site. X-ray crystallographic studies have shown that the antigen binding site is a pocket with an area of about 7nm^2 (Amit et al, 1990). Antibodies to 2-phenyloxazolane possess an interacting area of about 4nm^2 and all have a low affinity constant never exceeding 10^5M^{-1} (Alzari et al, 1990).

The interaction of antibodies with proteins has recently been reviewed by Davies and Cohen (1996). Antibody-antigen crystal structures demonstrate good shape complementarity between the interacting surfaces and the juxtaposition of polar residues permitting hydrogen bonding between antibody, antigen and water molecules in the binding site.

Recent studies of antibody amino acid composition show a clustering of aromatic residues at the antibody binding site (Jones and Wells 1996). Non-polar interactions may help explain why antibodies were generated with a higher affinity to S-PMA (MW 239) than to 2-HEMA (MW 220)

CHAPTER THREE

MONOCLONAL ANTIBODY PRODUCTION

INTRODUCTION

3.1 MONOCLONAL ANTIBODY PRODUCTION

The production of specific lymphocyte-myeloma cells has proved to be an effective means of generating antibodies to a wide range of antigens. As a result, hybridoma technology has become routine in many laboratories. Monoclonal antibody production involves many steps (figure 3.1) which have been performed in many different ways, and a number of extensive reviews exist (Goding, 1980 and 1986; Fazekas De St. Groth and Scheidegger, 1980; Galfre and Milstein 1981; Edwards 1981; Siddle 1985;). A critical step is the ability to achieve a high efficiency of hybridoma production upon fusion of sensitised lymphocytes with myeloma cells. This involves several distinct stages: 1) choice of myeloma cell line; 2) fusion of immunogen primed lymphocytes with myeloma cells; 3) culture and growth in selective medium; and 4) cloning and mass production of antibody in vivo or in vitro.

3.2 CHOICE OF MYELOMA CELL LINE

In general, the fusion of cells from different species does not give stable hybrids and the choice of myeloma cell depends upon the choice of animal used for immunisation. Immunisations (chapter 2) are usually carried out using Balb/c mice and the chosen myeloma cell line is therefore of Balb/c origin. The chain composition of the myeloma cell is also an important consideration in the choice of myeloma cell line. Kohler and Milstein (1975) used a mouse myeloma cell line called P3-X63/Ag8 derived from a Balb/c mouse MOPC-21.

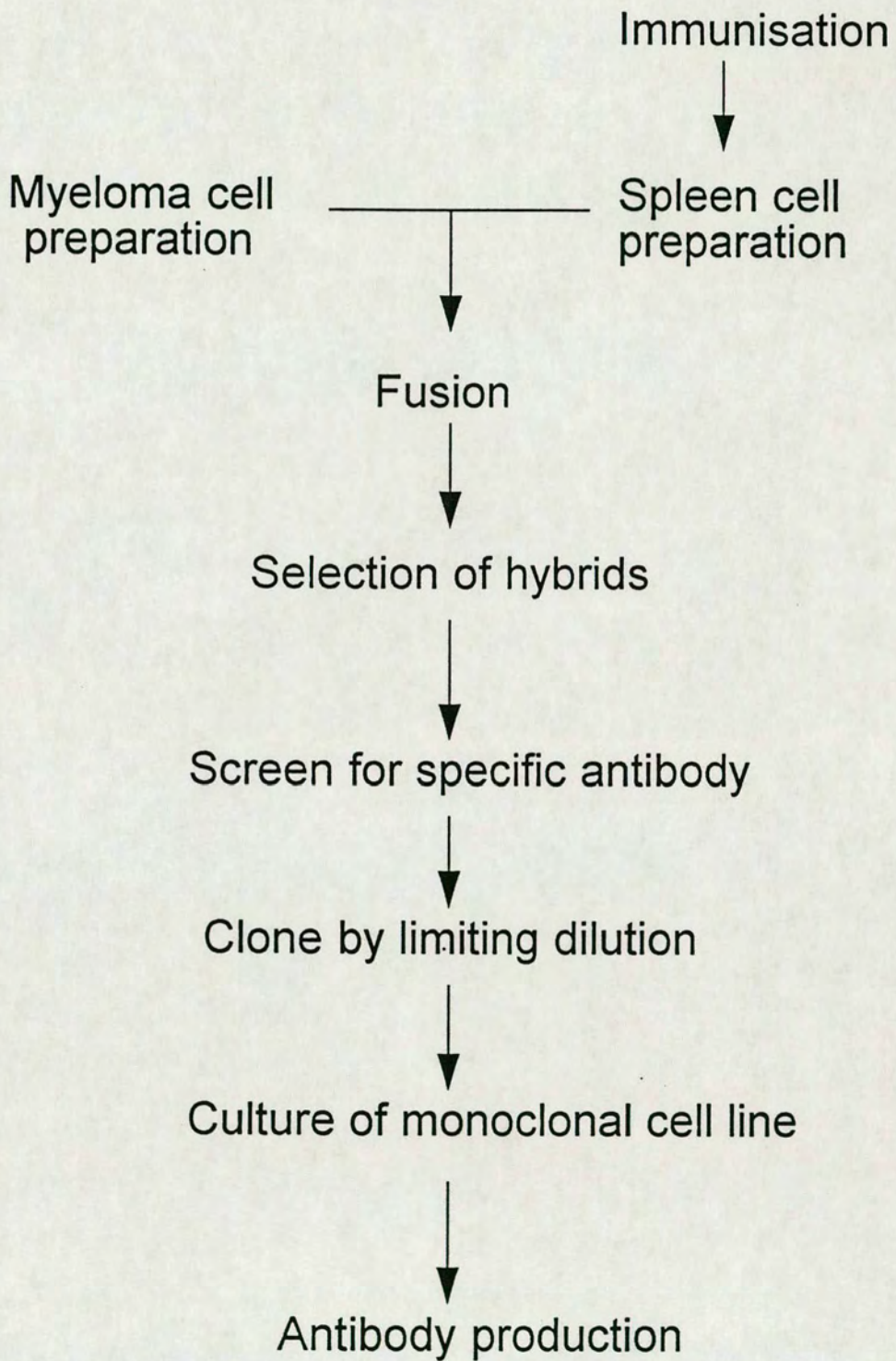


Figure 3.1 Overview of Monoclonal Antibody Production

P3-X63/Ag8 secretes IgG whose heavy and light chains are generally designated G(gamma) and K(kappa). When P3-X63/Ag8 fuses with a lymphocyte (whose light and heavy chains are designated L and H) a hybrid of composition HLGK is formed. Hybridoma cells co-dominantly express the immunoglobulin chains of both parental cells. The association of the chains is a random event and only a small number of immunoglobulins will express the heavy and light chains of the spleen cell (1 in 16). The majority of antibodies will therefore be inactive. In addition HLGK hybrids have a tendency to lose chromosomes. This loss is not random and the heavy chains are usually lost first.

A number of cell lines have been derived from P3-X63/Ag8. The variant P3-NS1-Ag4/1 synthesises Kappa light chains but does not secrete them. As a result, 25% of the immunoglobulins will be of spleen origin. Two myeloma cell lines X63-Ag8.653 (X63's) and Sp2/0. Ag8 (Sp2's) completely lack the ability to make their own IgG (Shulman et al 1978) and all the immunoglobulins secreted will therefore be of spleen origin. Thus the Sp2 myeloma cell line was selected for use.

3.3 FUSION

The spontaneous fusion of cells in nature is a rare event. However, almost any two cells can be made to fuse by the addition of a fusogen. Kohler and Milstein used inactivated Sendai virus as the fusogen in their original work. Polyethylene glycol MW 500-4000 (PEG) is now the agent of choice and is used in nearly all hybridoma work. The use of PEG increases the number of hybridoma cells when

compared with fusions performed with Sendai virus (Gefter et al 1977). Surprisingly, the concentration of PEG needed for a successful fusion is close to its toxic concentration (30-50%). However, even with the use of PEG, cell fusion is still a rare event, and fusion efficiency has been estimated by Siddle (1985) to be 1 in 10,000 cells.

After much investigation the fusion mechanism is still poorly understood. PEG appears to promote the close apposition of cell membranes while additives (antioxidant and/or polymerisation agents) in the commercial preparations may stimulate the fusion process. Variations in the additives and impurities may explain the differences in the fusion efficiencies achieved with different brands, lots and molecular weights of PEG. Although PEG encourages membrane and protein aggregation, transfer of the cytoplasmic contents appears to take place when the PEG is diluted (Wojcieszyn et al 1983). The transfer of the cytoplasmic contents of one cell to another results in the formation of a heterokaryon, a cell which possesses two or more nuclei. At the next division the nuclei of the heterokaryon fuse and a hybrid cell results.

Fusion protocols vary widely. The spleen to myeloma cell ratio does not appear to be critical within the ratios of 1:1-1:10 (spleen : myelomas) However the condition of the myeloma cells is critical to the success of the fusion. For the best fusion results the myeloma cells should be maintained in the logarithmic phase of growth for one week prior to fusion (Galfre and Milstein 1981). At fusion the myeloma cells should be in the rapid exponential phase of growth for optimum results. Spleen cells should be stimulated 3-4 days before

fusion when antigen induced proliferation is strongest.

The lymphocytes and myeloma cells are mixed and brought into close contact by centrifugation. PEG is added to the pelleted cells then slowly diluted by the addition of medium. PEG of MW's 500-4000 daltons at a concentration of 30-50% w/v are typically used and the variation does not appear to be critical. The use of PEG below 30% results in a low fusion efficiency whilst above 50% toxicity is a problem (Goding 1980, Lane 1985). The fusion procedure described in the work below is a modification of the method of Gefter et al (1977). Alterations in the original protocol are due in part to Fazekas De St.Groth and Scheidegger (1980) and Lane (1985).

3.4 CULTURE AND GROWTH IN SELECTIVE MEDIUM

After fusing, the hybrid cells are vastly outnumbered by the unfused myeloma and spleen cells. The spleen cells cannot grow indefinitely in culture and die within about five days. The myeloma cells, however, can grow indefinitely in culture and it is necessary to isolate a relatively small number of hybridoma cells from the large excess of myeloma cells. This can be achieved with the use of a selective medium which inhibits the growth and causes the death of the myeloma cells. The most popular strategy is that of Littlefield (1966).

The biosynthetic pathway for purines and pyrimidines can be blocked by the folic acid antagonist aminopterin. If this pathway is blocked in normal cells the DNA precursors are synthesised by an alternative route - the salvage pathway - in which preformed nucleotides are

recycled. The salvage pathway depends upon the enzymes thymidine kinase (TK) and hypoxanthine guanine phosphoribosyltransferase (HGPRT). If one of these enzymes is absent DNA synthesis ceases (Figure 3.2).

Mutant myelomas lacking the enzymes TK or HGPRT have been produced by the use of toxic drugs. TK⁻ mutants are difficult to induce since two simultaneous mutations, a rare event, are needed. HGPRT⁻ mutants are relatively easy to induce since the enzyme is coded on the X-chromosome. Mammalian cells possess only one active X-chromosome and only a single mutation is needed for total loss of the enzyme.

Using the strategy of Littlefield, mutant myeloma cells are chosen which are deficient in the enzyme HPGRT. The HPGRT⁻ myelomas are unable to grow in HAT selective medium, which contains the nucleotides hypoxanthine and thymidine and the antagonist aminopterin. The spleen cells are HPGRT⁺. The fusion of a HPGRT⁺ spleen cell with a HPGRT⁻ myeloma cell results in the formation of a HPGRT⁺ hybridoma cell. The hybridoma cell is able to grow in the HAT selective medium (Figure 3.3).

3.5 CLONING AND MASS PRODUCTION OF ANTIBODY

Following fusion, initial cell growth takes place in HAT selective medium. In general, antibody secreting cells grow slower than non-secreting hybrids and the non-secretors soon dominate the culture. Cloning, or the isolation of single desired hybridoma cells, should therefore be carried out as early as possible. The early detection of positive wells is essential for success.

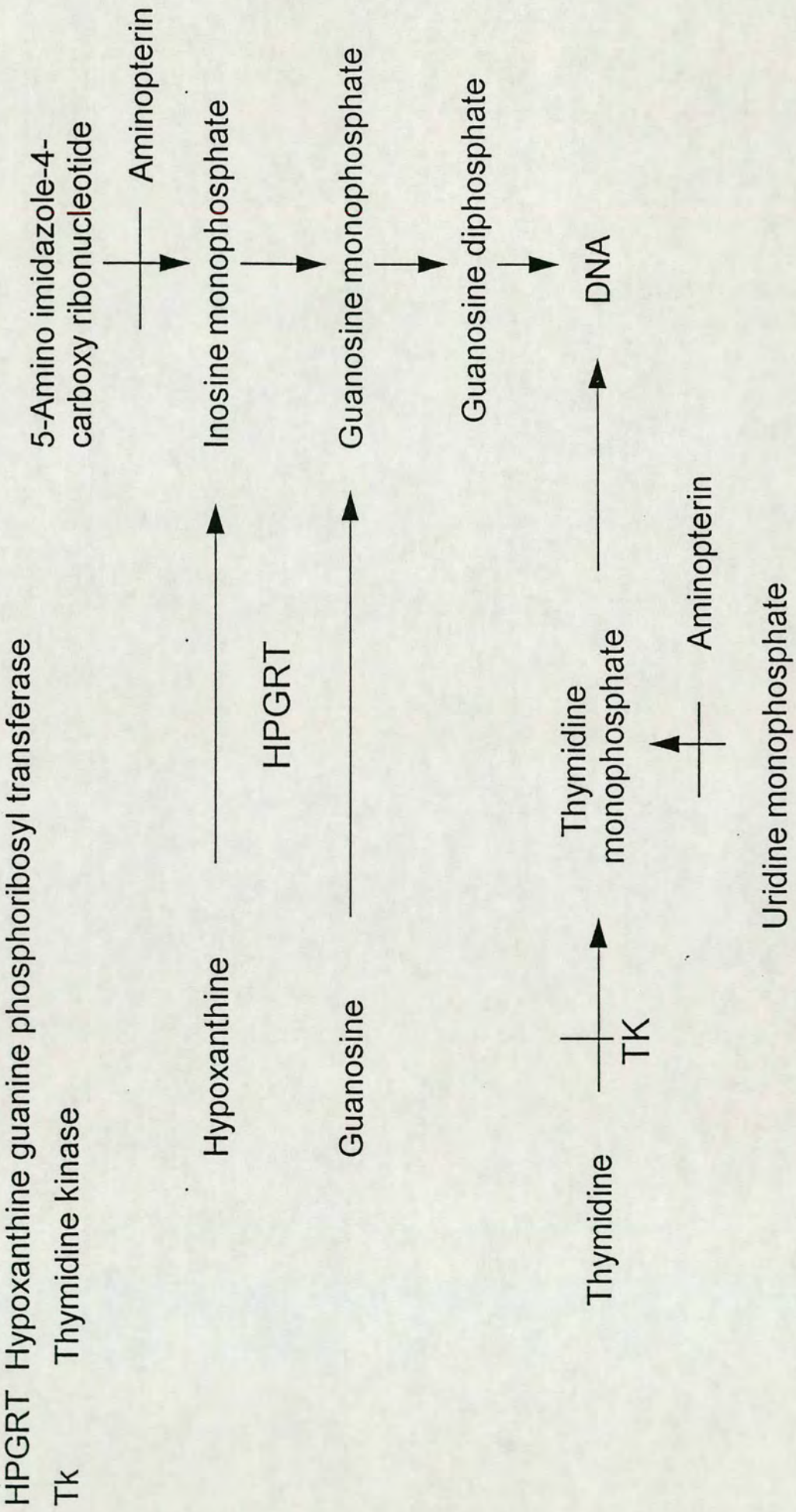


Figure 3.2 Biosynthetic and Salvage Pathway of DNA Synthesis

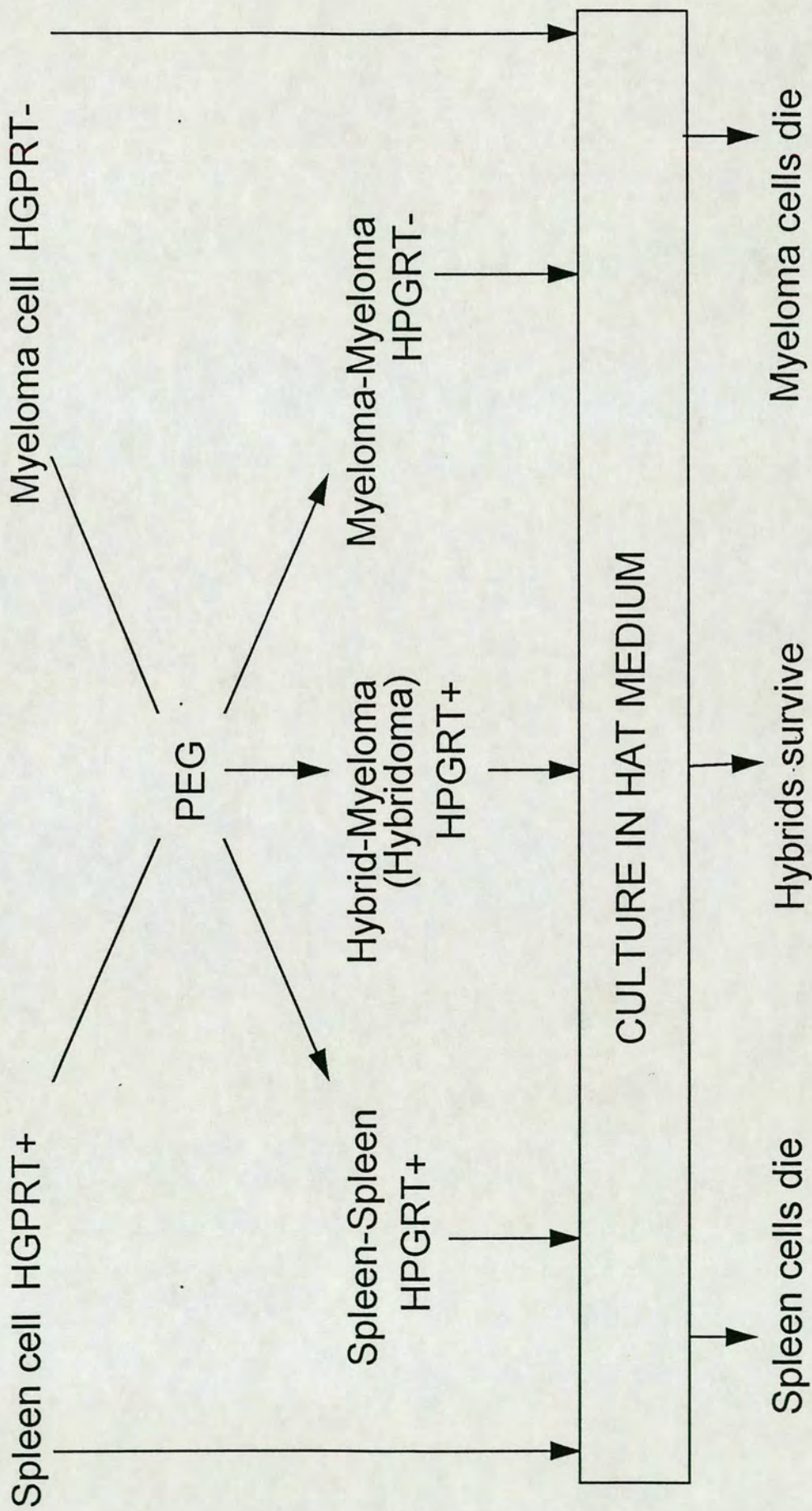


Figure 3.3 Post-fusion Cell Selection

The screening assay needs to be sensitive, detecting antibody at concentrations of 1ug/ml or less in supernatant. The assay should also be simple, giving rapid answers on many samples, but need not be precisely quantitative or totally specific at this stage.

Kohler and Milstein (1975) cloned by low dilution in semi-solid agar. The agar physically separates the cells until they form colonies large enough to be individually transferred to culture wells. Cloning by limiting dilution is now the usual method of choice.

Failure to obtain positive clones from strongly positive cultures may reflect the low proportion of positive cells or, the inherently poor cloning efficiency of some cells which will not grow well at low concentrations. To support the growth of single cells during cloning, feeder cells are routinely used in hybridoma technology. Peritoneal macrophages, splenocytes and thymocytes have all been used to support the growth of hybrids.

Having confirmed monoclonality, cell numbers are expanded by culture in stationary flasks. The harvested supernatant usually contains 10-100ug of antibody per millilitre of culture supernatant.

3.6 CHARACTERISATION OF ANTIBODY CONTAINING SUPERNATANT

Antibody titre is determined by serial dilution of the supernatant. The titre is the dilution of the supernatant which gives half the maximal binding activity. Titre is therefore a function of antibody concentration and avidity.

Antibody affinity can be determined by scatchard analysis. Here, antigen binding to a limiting concentration of antibody is expressed as a function of antigen concentration. It is often stated that monoclonal antibodies are of low affinity (10^8 - 10^9M^{-1}) compared to the best available polyclonal antisera (10^{11} - $10^{12}M^{-1}$). The isolation of low affinity antibodies reflects their preponderance in-vivo. Although high affinity antibodies are a minority, the production of antibodies with dissociation constants of 10^{10} - $10^{11}M^{-1}$ should be possible. Relative antibody affinity can be determined by the preparation of calibration plots.

Antibodies are divided into five major classes of immunoglobulin - IgM, IgG, IgA, IgE and IgD (Goding, 1986). Antigenic analysis of IgG myelomas has shown that they can be further grouped into four subclasses (eg. IgG's1-4). The differences all lie in the heavy chain of the immunoglobulin molecule and these are termed isotypic variants. Variations exist in the biological and physical properties of the antibodies in each class or isotype.

3.7 IN-VITRO IMMUNISATION

Although the production of monoclonal antibodies in-vivo has become a routine technique it does not always lead to the production of specific antibody secreting hybridomas. For example, 2-HEMA is a small, weakly immunogenic molecule (chapter 2) and a poor antigen in-vivo. To improve the efficiency of specific hybridoma production, in-vitro immunisation with 2-HEMA has been examined.

A primary antigen specific stimulation of dissociated mouse spleen

cells in-vitro was first demonstrated by Mishell and Dutton in 1966. Using sheep red blood cells (SRBC's) as the immunogen an in-vitro response comparable to that in-vivo was obtained. Twelve years later, again using SRBC's as the immunogen, Hengartner et al (1978) combined in-vitro immunisation with hybridoma technology for the production of monoclonal antibodies. Luben and Mohler (1980) combined in-vitro immunisation with hybridoma methodology to produce antibodies to a soluble protein antigen (Lymphokine Osteoclast Activating factor) and found in-vitro immunisation to be three times more effective at producing hybridomas of the required specificity than in-vivo immunisation. However in-vitro immunisation has not yet become a widely used procedure and the theoretical advantages of in-vivo immunisation are being realised only slowly in practice.

In-vitro immunisation requires smaller quantities of antigen (0.1-200ug) and labile antigens may be sufficiently stable in-vitro to allow sensitisations which would be impossible in-vitro. Responses to weakly immunogenic antigens in-vitro, conserved epitopes and self-antigens may also be possible (Borrebaeck and Muller 1986), due to an absence of immunosuppression mechanisms in-vivo (Miner et al 1981). Each step of the procedure can be monitored and the time (3-5 days) is much shorter than the time taken for an in-vivo immunisation (typically 28 days). The number of antibody producing cells per 10^6 spleen cells is significantly higher in-vitro due to the preferential survival of in-vitro activated cells. Borrebaeck 1986) has reviewed the progress of in-vitro immunisation and monoclonal antibody production. Several in-vitro immunisation procedures have been described for the production of antigen

specific hybridomas (Reading 1986, Boss 1986).

B-cell activation appears to consist of three separate stages- 1) induction/activation, 2) proliferation and 3) differentiation, requiring the co-operation of T-helper cells, accessory cells (eg. macrophages, monocytes) and B-cells. Their interactions are regulated by membrane bound proteins (MHC, class II molecules) and by soluble antigen non-specific growth and differentiation factors (the lymphokines). The cell-cell interactions which characterise the in-vivo immune response are absent in laboratory cultures but the soluble growth and differentiation factors must be added in the form of conditioned media. The conditioned media used in in-vitro immunisation appear remarkable in their heterogeneity. Thymocyte conditioned media (Luben and Mohler 1980, Van Ness et al 1984 and Ossendorp et al 1986), mixed lymphocyte culture media (Miner et al 1981 and Pardue et al 1983) and the supernatant from phorbil ester activated thymoma cells EL-4 (Borraboeck and Moller 1986 and De Boer et al 1988) have all been used to stimulate the development of antigen specific hybridomas.

An additional consideration when setting up an in-vitro immunisation is the source of serum. Rathjan and Underwood (1985) found batch to batch variation in calf serum critically influenced the hybridoma yield, possibly due to variations in the levels of endotoxins or hormones. Calf serum also gives high levels of non-specific hybridomas due to the activation of B cells by serum proteins. A number of authors including Mullbacher et al (1985) have reported the use of rabbit serum at 1-3% which will reduce the effect of serum proteins when compared to 15% calf serum. The effect of serum

proteins could be avoided by carrying out the entire procedure under protein free conditions, and several authors have described conditions for protein free in-vitro immunisations (Ossendorp et al 1986, De Boer et al 1989).

Antibodies secreted by hybridomas derived from in-vitro immunised spleen cells are predominantly of the IgM isotype, and this is thought to represent the primary response of activated naive B cells. IgG antibodies, however, are the result of the activation of antigen specific memory B cells formed after a first exposure to antigen. Takakashi et al (1987) increased the number of hybrids secreting IgG to 50% by prolonging the stimulation period from 5 to 8 days. Generally the increasing cell death beyond 5 days in culture has resulted in very poor fusion efficiencies. De Boer et al (1987) reported in-vivo priming followed by in-vitro boosting increased the number of IgG secreting hybridomas from 10% to 85%, successfully combining the advantages of both procedures.

This chapter describes the production of monoclonal antibodies which recognise S-PMA and 2-HEMA. Antibodies which bind S-PMA were derived from lymphocytes sensitized in-vivo. High affinity antibodies to 2-HEMA were difficult to induce in-vivo (Chapter 2), and in-vitro immunisation was therefore examined. The relative affinity and specificity of the antibodies produced were determined in characterisation studies. The results of the fusions and the characterisation studies will be discussed.

METHODS AND MATERIALS

MATERIALS

PLASTIC WARE

24 and 96 well tissue culture plates, and Immunoplates Maxisorb f96 cert were purchased from Nunc (Gibco Ltd. Paisley, Scotland.)

25cm² and 75cm² sterile 0.2µm filter vented culture flasks and Transtar 96 well transfer cartridges were purchased from Costar (High Wycombe, Oxfordshire).

1, 10 and 20ml sterile syringes (Becton Dickenson), sterile blind hubs (Sabre), sterile Kwillis (Avon Medicals), 30ml sterile universal container (Sterilin), sterile petri dish (Greiner), 60 and 200ml sterile square media bottles (Nalgene), sterile disposable scalpels (Swann-Morton). 0.6 x 16mm and 0.8 x 16mm Monoject™ hypodermic needles (Sherwood Medicals).

CULTURE MEDIA

The following media constituents were purchased from Flow Laboratories Ltd. (Rickmansworth, Herts.):-

1x RPMI 1640 Medium containing 2.0g/l sodium bicarbonate. Sodium Pyruvate (100mM, 11.0mg/ml), L-glutamine (200mM, 29.23mg/ml). 50x HAT solution containing 5000uM hypoxanthine, 20uM aminopterin and 800uM thymidine. 50x HT solution containing 5000uM hypoxanthine, 800uM thymidine and x100 amino acids for basal medium (modified).

Antibiotics:- Pencillin (5000Iu/ml) Streptomycin (5000ug/ml), Amphotericin B Deoxycholate (Fungizone [250ug/ml]), and Gentamicin Sulphate (50ng/ml).

Myoclone Plus Foetal Calf Serum and Protein Free Serum Free Medium (Gibco Ltd.), DMSO (tissue culture grade), mercaptoethanol and tryptan blue (Sigma Chemical Co. Ltd.), Polyethylene glycol (PEG 1500; Sera-Lab, Crawley Down, Sussex)

RPMI 1640 was stored at 4° C. All other media additives were stored in aliquots at -20° C. All media were stored at 4° C. and used within 2-3 days of preparation.

1. STANDARD SERUM FREE MEDIUM:-

Standard serum free medium consisted of RPMI 1640, penicillin (100Iu/ml) and streptomycin (100ug/ml).

2. COMPLETE MEDIUM:-

Consisted of serum free medium with L-glutamine (2mM), sodium pyruvate (1mM), 10-15% v/v myoclone foetal calf serum and Fungizone (2.5ug/ml).

3. SELECTION MEDIA:-

i) HAT medium consisted of complete medium with a 1/50 dilution of stock HAT solution. The final concentrations were hypoxanthine 100um, aminopterin 0.4um and thymidine 16um.

ii) HT medium consisted of complete medium with a 1/50 dilution of stock HT solution. Final concentrations were hypoxanthine 100um and thymidine 16um.

4. FREEZING MEDIUM:-

Freezing medium consisted of 90% v/v foetal calf serum 10% DMSO.

5. IN-VITRO IMMUNISATION MEDIUM:-

In-vitro immunisation medium consisted of 50ml of protein free serum free medium containing 2ml glutamine, 1ml sodium pyruvate, 1ml of HT, 1ml of non-essential amino acids, 2ml of sodium bicarbonate, 35ul of mercaptoethanol, 0.5ml of fungizone and 2.5ml of myoclone plus FCS (5%).

MYELOMA CELL LINES

Balb/c myeloma cell line Sp 2/0. Ag 1-4, which secretes no immunoglobulin chain, was purchased from Flow Laboratories. Myeloma cells were cultured in complete medium. Aliquots of cultured cells (10^6 cells in 500ul of freezing medium) were stored frozen at -170° C. in liquid nitrogen.

METHODS

All cell culture procedures were carried out under sterile conditions using aseptic technique. All cells were cultured in a 37°C humidified incubator in the presence of 5% CO₂ in air.

3.8 PREPARATION OF MYELOMA CELLS

Thawing Cells

Myeloma cells (10⁶ cells in 500ul freezing medium) were stored in cryotubes frozen at -180°C in liquid nitrogen. DMSO is toxic to thawed myeloma cells therefore the following operations were carried out rapidly. A cryotube containing a frozen solution of myeloma cells was thawed in a water bath at 37°C., sprayed with 70% alcohol:water and dried. The cells were transferred to a 30 ml plastic pot (Sterilin) and diluted with 20 mls of cold serum free medium inside a laminar flow hood. Diluted cells were centrifuged (1500rpm., 5min.) and the supernatant discarded. The remaining pellet was gently resuspended in 10mls of warm complete medium. Resuspended cells were cultured in a 25cm² 2um vented culture flask.

Culture of Myeloma cells.

Myeloma cells were grown in stationary culture and maintained in a logarithmic phase of growth. Cells were cultured initially in 25cm², 0.2um vented culture flasks and, as the single layer of cells approached confluency, expanded to 75cm² vented culture flasks. Cell medium (complete) was changed at regular intervals (approximately every 2-3 days). 2-5 x 10⁷ cells were required per

fusion, approximately three 75cm² culture flasks. Viability was determined by Trypan Blue staining and cells were counted using a Neubauer haemocytometer.

Immediately prior to fusion, myeloma cells were resuspended, by gently scraping the bottem of the culture flask, and the suspension sedimented by centrifugation (1500rpm., 5min.). The pellet was resuspended in 10ml of 37⁰ C. serum free medium.

3.9 PREPARATION OF SPLEEN CELLS.

On two consecutive days prior to fusion the mouse was immunised intra-peritoneally with 100ug of conjugate in 100ul of PBS. Two days after the final pre-fusion immunisation the mouse was sacrificed by asphyxiation in diethyl-ether. The mouse was placed on its right hand side and soaked with 70% alcohol:water. The spleen was carefully removed using a sterile pair of scissors and forceps and placed in a sterile container containing 20mls of cold serum free medium.

The spleen was transferred to a petri dish and any remaining connective or fatty tissues cut away. A superficial cut was made along the length of the dorsal ridge of the splenic capsule with a scalpel. Two 10ml syringes fitted with 0.6 x 16mm gauge needles and filled with serum free medium at 37⁰ C, were used to anchor the ends of the spleen and flush the splenocytes out of the capsule. This yielded a high population (10⁸) of single cells in suspension. The cell suspension was centrifuged, the medium discarded, and the pellet resuspended in 10ml of 37⁰ C. serum free medium.

3.10 FUSION PROTOCOL.

The fusion procedure employed was based on a modification of a method described by Lane (1985). The splenocytes and myeloma cells prepared above were mixed, centrifuged and the pellet resuspended in 20ml 37⁰ C. serum free medium. The mixed cell suspension was divided equally between six 30ml universal containers, and the cells pelleted by centrifugation. The supernatants were discarded and excess fluid carefully drained so as not to dilute the polyethylene glycol (PEG 1500) to be added in the next stage. Each pellet was then gently disrupted, using the needles (0.6 x 16mm) fitted to 1ml syringes. PEG-1500 (0.3ml/universal, 37⁰ C.) was added evenly, with gentle agitation, over 30 seconds. The PEG was immediately diluted by the addition of 10ml of warm serum free medium added gradually over 1 minute. The cells were centrifuged, resuspended in 6ml of complete culture medium (37⁰ C.) and distributed evenly (100ul/well using a 10ml syringe fitted with a 0.8 x 16mm needle) between the central 60 wells of a 96 well tissue culture plate. Finally, double strength HAT medium (100ul/well) was added and the culture plates incubated as described.

Every two days 100ul of medium was removed from the wells and replaced with 100ul fresh HAT medium. After twelve days, HAT medium was replaced with HT medium. The appearance of hybridoma clones observed microscopically and indicated macroscopically by a change in medium colour, from pink to yellow, usually occurred between days 5-12.

3.11 SCREENING CULTURE SUPERNATANTS

3.11.1 BY ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

Coating Plates

Haptens conjugated to Bovine Serum Albumin (BSA) were used for plate coating. A solution of conjugate in coating buffer was added (100ul) to the central 60 wells of a microtitre assay plate. The plate was sealed with a plastic cover and incubated for 3 hours at room temperature. The wells were emptied and washed 5 times with saline/Tween solution, shaken dry and stored at 4° C. until use.

Screening Assay

Supernatants were transferred to the antigen coated wells of a microtitre plate using a 96 well transfer cartridge. The plate was sealed with a plastic film and incubated overnight at room temperature. The wells were emptied, washed 5 times with saline/Tween and shaken dry. Excess wash solution was removed by aspiration. To each well was then added 100ul of anti-mouse IgG peroxidase conjugated antibody diluted 1:1000 in PBS/Tween. The plate was covered and incubated for a further 2 hours at room temperature. The plate was washed again and dried, as above, and 100ul of ABTS substrate added. In the presence of peroxidase this substrate turns from a colourless to a green solution. Optical density was measured at 405nm using a microtitre plate reader (spectrophotometer).

The capacity of an antibody to recognise free hapten was tested in competitive inhibition studies. Supernatant was diluted to give a

limiting dilution in PBS/Tween and transferred (50ul) to the wells of a conjugate coated plate. The supernatant was incubated in the presence of either standard or PBS/Tween (50ul). The assay was performed as described above.

3.11.2 BY RADIOIMMUNOASSAY (RIA)

Wells from the 2-HEMA fusions which gave a positive result in the initial ELISA screening assay were also tested by RIA. RIA conditions have been previously described (chapter 2). Separation of bound and free label was achieved with 5% dextran coated charcoal (which has been described) and saturated ammonium sulphate solution.

Separation of Bound and Free Label with Saturated Ammonium Sulphate

To the samples and the blanks was added 50ul of normal rabbit serum and 450ul of saturated ammonium sulphate solution. The tubes were mixed, incubated at room temperature for 20 minutes and centrifuged (2400rpm, 4°C for 20 minutes). The supernatant was removed by aspiration and discarded. The pellet was resuspended in 200ul of PBS and transferred to a scintillation vial. Scintillant was added (5ml) and the radioactivity assessed using a beta radiation counter. The counts are directly proportional to the amount of labelled analyte bound by the antibodies.

3.12 CELL CLONING BY LIMITING DILUTION

Preparation of thymocyte feeder cells

A young (4-6 weeks old) Balb/c mice was sacrificed by ether anaesthesia, placed on its back and soaked with 70% alcohol:water. An incision was made in the mid-line of the thorax below the rib cage and extended longitudinally to the neck region using a sterile pair of scissors. The thoracic cavity was opened using sterile scissors and forceps. The thymus, a two lobed whitish-cream coloured organ anterior to the heart, was carefully removed and transferred to 25mls of cold serum free medium in a sterile container. The thymus was then transferred to a petri dish containing 10mls of serum free medium and teased with a pair of blunt forceps. The resulting cell suspension was transferred to a 30ml sterilin, discarding the connective tissue, and centrifuged at 1500rpm for 5 minutes. The medium was discarded and the pellet resuspended in 20ml of warm complete medium. This method releases $2-3 \times 10^8$ thymocytes, in general, from a thymus.

Cloning by limiting dilution

It is most likely that positive wells in the post fusion screening assay will contain clones originating from more than 1 parent cell. The aim of this dilution procedure is to achieve 1 hybridoma cell per well which develops to form a single hybrid clone.

Hybridoma cells were diluted (50 cells per ml) in 4mls of thymocyte containing medium. 2.4ml of this suspension were equally distributed over the first 2 rows (24 wells) of a 96 well microtitre plate.

Theoretically, this gives 5 cells/well. To the remaining 1.6ml was added 2.4ml of thymocyte containing media, and 2.4ml of this were equally distributed over the next two rows of the microtitre plate (2 cells/well). The above step was repeated a second time to give, theoretically, 1 cell per well. Finally, to the remaining 1.6ml was added 0.8ml of thymocytes and the 2.4ml was distributed over the last 2 rows of the microtitre plate, to dispense 1 cell in every 2 wells. Warm complete medium (150ul) was added to all the wells. Plates were screened approximately 10-15 days later.

3.13 FREEZING CELLS

Freezing 96 cell plates

Medium was carefully removed from the wells of a tissue culture plate, leaving the remaining cells adhered to the well surface. Cold freezing medium (kept on ice prior to use) was added to the wells (100ul/well) and the plates sealed. Sealed plates were immediately placed in a jiffy bag and transferred to a -70° C. freezer.

Freezing larger numbers of cells

Cells in 2ml wells and tissue culture flasks were resuspended in the culture medium, transferred to a 30ml universal container and the numbers determined by counting a sample using a Neubauer haemocytometer. The cells were sedimented by centrifugation (1500rpm., 5min.), the medium discarded and the cells resuspended in cold freezing medium (stored on ice prior to use) at a density of

10^6 cells/1 ml medium. Aliquots (500ul/cryotube) were frozen to -170°C . in liquid nitrogen using a programmable ramp freezer. The temperature was reduced in 2 stages, stage 1 at $-1^{\circ}\text{C}/\text{min}$ down to -70°C and stage 2 at $-10^{\circ}\text{C}/\text{min}$. down to -140°C . Frozen cells were transferred to a liquid nitrogen cell store and kept at -170°C .

3.14 IN-VITRO IMMUNISATION

The donor mouse was sacrificed by asphyxiation in diethyl-ether and the spleen carefully removed as described above. A suspension of single spleen cells was obtained by perfusion with 20ml of protein free serum free medium. Perfusion of the spleen is described in detail above. The cell suspension was centrifuged, the medium discarded and the cells resuspended in 10ml in-vitro immunisation medium. The spleen cells were transferred to a 25ml tissue culture flask.

To the flask was added 10ml of thymocyte conditioned medium, 200ul of BRM and filtered antigen (0.75-1ug/ml). Antigen was diluted in protein free serum free medium and filtered through a low protein binding filter 0.2um. The flasks were cultured in a 37°C . humidified incubator in the presence of 5% CO_2 in air for five days.

Immediately prior to fusion the cultured cells were resuspended and the suspension sedimented by centrifugation (1500rpm., 5min.). The pellet was resuspended in 10ml of serum free medium. Viability was determined by Trypan Blue staining and cells were counted using a Neubauer haemocytometer. Cells were fused, cultured and screened as described above.

3.15 ANTIBODY CHARACTERISATION

Relative antibody affinity was determined by the preparation of calibration plots. The lower limit of detection was determined for each supernatant under optimal assay conditions. Antibody specificity was determined in cross-reactivity studies. Calibration plots were prepared using compounds containing structurally similar features, and the IC₅₀ values (the concentration of standard that gives 50% inhibition) determined.

3.16 FUSIONS

3.16.1 S-PHENYL-N-ACETYL-L-CYSTEINE

Fusions 1 and 2

Mice 3 and 1 immunised with phenylcysteine coupled to KLH with the homobifunctional crosslinker bis(sulfosuccinimidyl)suberate were selected for fusion (chapter 2). Mouse 3 was primed (100ug of conjugate in 200ul PBS) 29 and 30 days after the second challenge. Mouse 1 was primed 72 and 73 after the second challenge. Two days after priming the spleens were removed and the splenocytes fused with the myeloma cell line Sp2/0. Ag1-4 (day 0). The fused cells were cultured in HAT media (days 0-12), HT media (days 13-19) and complete media thereafter. Supernatants were screened by ELISA using plates coated with BSA-BS³-PMA (1ug/ml) on day 9. The number of wells containing hybridoma clones were determined by microscopic examination on the day of screening.

Rapidly growing clones which were positive in the screening assay

were expanded into 24 well tissue culture plates. All the positive wells and the expanded wells were re-screened on day 11. In the second screen, competitive inhibition studies using free S-PMA (100ug/ml in PBS/Tween) were included. Cells from wells (96 well plates) containing antibody which bound free hapten were cloned by limiting dilution. Clone plates were screened 14 days later for the presence of antibody activity and inhibition studies were carried out. Monoclonal wells were determined on the day of screening by microscopic examination.

3.16.2 S-(2-HYDROXYETHYL)-N-ACETYL-L-CYSTEINE

Fusions 3-17 (in-vivo immunisations)

Mice immunised with 2-HEMA coupled to either KLH, gelatin or MSA were primed 6 to 22 weeks after the second challenge. Two days later the spleens were removed and fused. The fused cells were cultured as above. The plates were screened by ELISA on day 14. Supernatants from the KLH-ABS-HEMA and GEL- β ALA-HEMA immunised mice were screened against plates coated with BSA-ABS-HEMA conjugate (0.5ug/ml in coating buffer). Supernatants from the KLH, GEL and MSA-7C-HEMA immunised mice were screened against plates coated with BSA-7C-HEMA (1ug/ml in coating buffer). The number of wells containing viable hybridomas were counted on the day of screening. Rapidly growing colonies which were positive in the screening assay were expanded into 24 well tissue culture plates. Positive and expanded wells were rescreened on day 16. In the second screen competitive inhibition studies with 2-HEMA (100ug/ml, 4.2×10^{-7} moles/ml in PBS/Tween) were

included. Positive wells were also screened by RIA against 3×10^{-11} (2000dpm/min) or 1.5×10^{-10} moles/ml (10000dpm/min) of label. Separation of bound and free label was achieved using either 5% dextran coated charcoal, or saturated ammonium sulphate solution.

Fusions 18-20 (in-vitro immunisations)

Study 1:-

Spleen cells from mouse 3 immunised with Gel-7C-HEMA were primed in-vitro 16 weeks after the first challenge. Spleen cells were cultured in the presence of 1ug/ml of Gel-7C-HEMA. The culture was examined daily macroscopically. After 5 days the cells were fused as described above. After 12 days the wells were screened by ELISA. Inhibition studies (100ug/ml, 4.2×10^{-7} moles/ml 2-HEMA) were performed on the positive wells two days later. Positive wells were also screened by RIA (3×10^{-11} moles/ml of 2-HEMA). Bound and free label was separated with saturated ammonium sulphate solution.

Study 2:-

In a comparative study a KLH-7C-HEMA immunised mouse was boosted in-vivo 12 weeks after the second challenge. Spleen cells from a second KLH-7C-HEMA immunised mouse were primed in-vitro (12 weeks after the second challenge), and a 6 week old naive Balb/c mouse was immunised in-vitro. In-vivo immunisations were with 200ul of 100ug/ml KLH-7C-HEMA in PBS. In-vitro immunised cells were cultured in the presence of 0.75ug/ml of KLH-7C-HEMA. On the day of fusion the number of viable cells was determined. The fusions were cultured and screened by ELISA and RIA (3×10^{-11} moles/ml).

3.16.3 MSA-ACETYLCYSTEINE IMMUNISED MICE

Fusions 21-23

Mice 1, 2 and 4 immunised with MSA-acetylcysteine were first primed for fusion 28, 65 and 66 days after the second challenge. All three mice were given the second pre-fusion boost on the following day and fused two days later. The fused cells were cultured as described above. On day 10 the supernatants were screened against plates coated with 0.1ug/ml of BSA-acetylcysteine conjugate. Positive screening wells were rescreened on day 12 in the presence and absence of free S-PMA (200ug/ml), 2-HEMA (173ug/ml) and acetylcysteine (136ug/ml). All three inhibitors contain 8.4×10^{-7} moles/ml of standard. Positive wells were also screened by RIA against 2-HEMA (1.5×10^{-10} moles/ml). Free and bound 2-HEMA was separated with 5% dextran coated charcoal.

RESULTS

S-PHENYL-N-ACETYLCYSTEINE

3.17 Fusion 1

Spleen cells from mouse 3 immunised with phenylcysteine conjugated to KLH were successfully fused with the myeloma cell partner Sp2/O's. On the day of screening 60% to 100% of the wells per plate contained 1 or more viable hybrid colonies (table 3.1). Supernatants were screened against plates coated with BSA hapten conjugate. There was considerable variation in the colour intensity of the wells and an O.D. of three times background (background = 0.05 units) was detected in 20% to 100% of the wells per plate. Wells with an O.D. greater than 1 were recorded as positive (table 1). All of the positive wells contained viable hybridoma clones. Binding of free hapten by these antibodies was detected in 85 supernatants (82% of the positive wells). Twenty three supernatants (22% of the positive wells) showed more than 40% inhibition with 100ug/ml of S-PMA (table 3.1).

TABLE 3.1 SCREENING RESULTS FOR PMA FUSION 1 PLATES 1-6

PLATE	WELLS PLUS HYBRIDS (%)	POSITIVE WELLS IN SCREEN*	POSITIVE WELLS IN INHIBITION STUDY**
1	100	34	3
2	90	7	1
3	100	11	2
4	60	0	0
5	100	16	3
6	100	36	14

* O.D. greater than 1 in screening assay

** 40% inhibition or more with 100ug/ml of S-PMA

These results were confirmed in a repeat assay two days later (Table 3.2). Well 6B2 demonstrated the greatest inhibition (79%) with S-PMA. The antibody did not recognise 2-HEMA. Well 6B2 was cloned and expanded as described in the methods. Monoclonality was confirmed for 6E2H6.

TABLE 3.2 FUSION 1 - INHIBITION STUDIES WITH 100ug/ml S-PMA AND 2-HEMA

POSITIVE WELL	% INHIBITION WITH	
	PMA	2-HEMA
1B2	52	-
1D4	49	-
1D9	42	-
2E8	61	23
3C11	69	8
3F8	69	-
5D10	56	-
5E10	70	-
5F6	55	-
6B2	79	-
6B4	55	15
6B7	52	18
6B8	44	8
6B11	54	11
6C11	71	21
6D11	46	-
6E8	64	-
6E10	56	6
6F2	44	11
6F4	42	-
6F6	48	-
6F10	47	7
6F11	54	5

3.18 FUSION 2

Spleen cells from mouse 1, immunised with phenylcysteine conjugated to KLH, were successfully fused with the Sp2 myeloma cell line. On screening, 90% to 100% of the wells per plate contained 1 or more

viable clones (table 3.3). The plates were screened by ELISA using the BSA conjugate as the coating material. Again there was considerable variation in the colour intensity of the wells, and 36% to 100% of the wells per plate gave O.D.'s greater than three times the background. Supernatants with an O.D. of greater than 1 in the screening assay were recorded as positive (table 3.3). All the positive wells contained viable hybrid colonies. Binding of free hapten was detected in 82% of the positive wells, and 22 supernatants (31% of the positive wells) gave more than 40% inhibition with 100ug/ml of S-PMA (table 3.3).

TABLE 3.3 SCREENING RESULTS FOR S-PMA FUSION 2 PLATES 1-6

PLATE	WELLS PLUS HYBRIDS (%)	POSITIVE WELLS IN SCREEN*	POSITIVE WELLS IN INHIBITION STUDY**
1	90	14	3
2	90	5	2
3	95	16	5
4	95	2	2
5	100	49	6
6	90	14	4

* O.D. greater than 1 in screening assay

** 40% inhibition or more with 100ug/ml of S-PMA

These results were confirmed in a repeat assay two days later (Table 3.4).

TABLE 3.4 FUSION 2 - INHIBITION STUDIES WITH 100ug/ml S-PMA

WELL INHIBITION(%)		WELL INHIBITION(%)	
1C11	46	4G11	51
1G7	86	5B4	59
1G11	52	5B6	56
2B4	75	5C7	65
2C3	51	5D4	50
2G10	64	5D10	57
3B2	54	5F10	55
3B5	53	6B2	60
3F9	93	6B8	81
3G9	87	6C2	50
4C2	74	6C9	52

Supernatants 1G7, 3F9, 3G9, 4C2, 6B2 and 6B8 demonstrated the greatest inhibition in binding studies with S-PMA. The relative affinities of the supernatants were assessed by comparing calibration plots developed using each supernatant. Supernatants 3G9 and 6B8 gave the lowest limits of detection. The detection limits were less than 0.1 and 1ug/ml of S-PMA respectively (table 3.5). Wells 3G9 and 6B8 were cloned and expanded.

Monoclonality was confirmed for wells 3G9F6 and 6B8F9. These wells were expanded into tissue culture flasks and the supernatant harvested.

TABLE 3.5 INHIBITION WITH 100UG/ML OF S-PMA AND THE LIMIT OF DETECTION IN A CALIBRATION PLOT DEVELOPED BY ELISA

WELL	INHIBITION (%)	LIMIT OF DETECTION
1G7	86	10ug/ml
3F9	90	10ug/ml
3G9	87	>0.1ug/ml
4C2	74	5ug/ml
6B2	64	5ug/ml
6B8	78	1ug/ml

3.19 COMPARATIVE STUDIES WITH MONOCLONAL SUPERNATANTS 6B2, 3G9 AND 6B8.

Assay conditions (eg. plate coating, antibody concentration) were optimised for each supernatant and S-PMA calibration plots prepared. The calibration plot developed with antibody 3G9 is shown in figure 3.4. The figure shows the mean of six replicate values and plus/minus one standard deviation. The lower limit of detection was 0.04ug/ml of S-PMA. The lower limit of detection corresponds to the intersection of the lower 95% confidence limit of the blank with the upper 95% confidence limit of the standard plot. The assay range for 6B2 and 6B8 are given in table 3.6.

TABLE 3.6 COMPARISON OF OPTIMISED ASSAY RANGE FOR SUPERNATANTS 6B2, 3G9 AND 6B8

SUPERNATANT	ASSAY RANGE(ug/ml)
6B2	10-200
6B8	0.2-10
3G9	0.04-2

Calibration plots were also prepared in urine. Calibration plots in PBS and urine developed with antibody 3G9 are shown in figure 3.5. The presence of urine does not appear to affect the performance of antibody 3G9. The preparation of calibration plots in urine did affect the performance of supernatants 6B2 and 6B8 (Table 3.7).

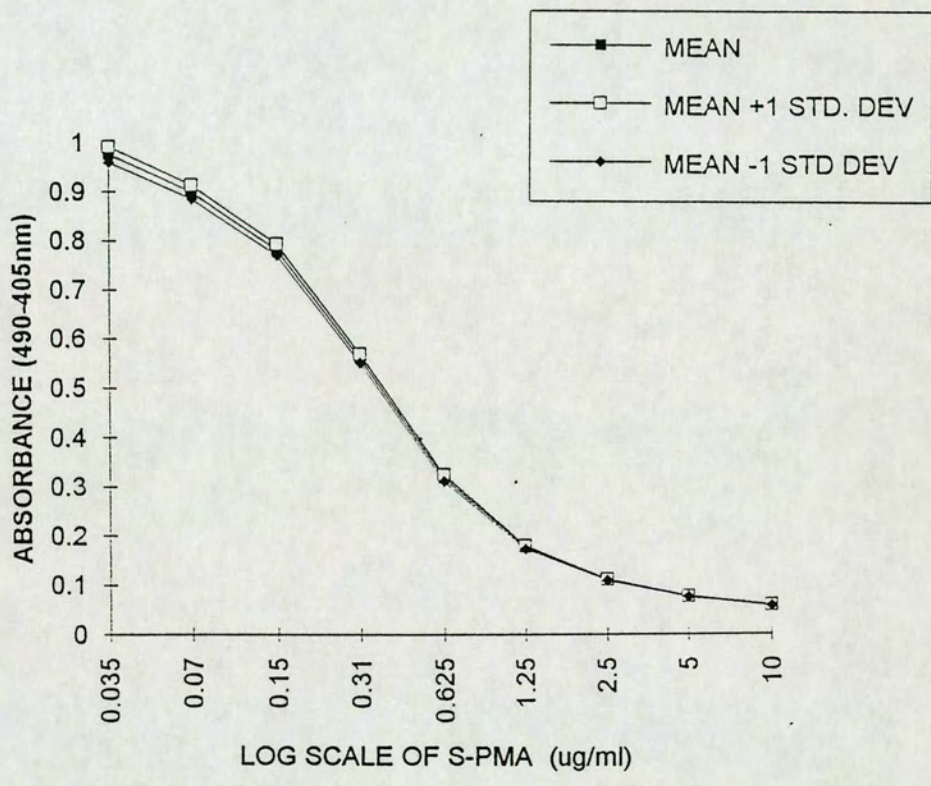


Figure 3.4 S-PMA calibration plot developed with antibody 3G9.

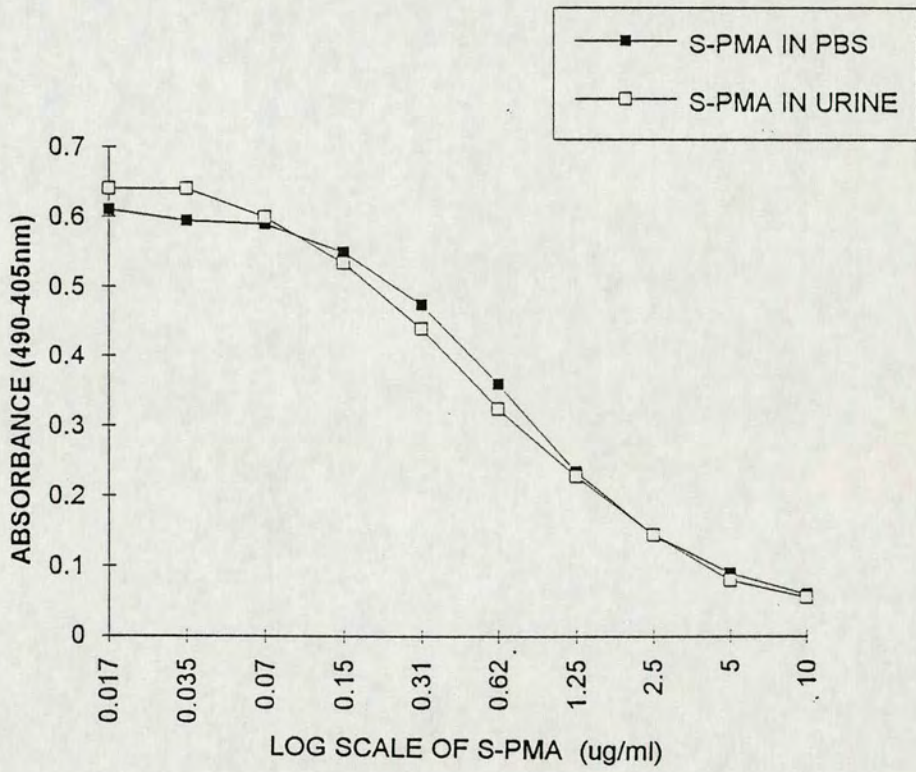


Figure 3.5 S-PMA calibration curves developed with antibody 3G9 in PBS and urine

TABLE 3.7 IC50 VALUES (ug/ml) OBTAINED WITH CALIBRATION PLOTS DEVELOPED IN URINE AND PBS

SUPERNATANT	IC50 (ug/ml)	
	PBS	URINE
6B2	60	<100
6B8	2.5	100
3G9	0.4	0.4

Cross-reactivities of 6B2, 6B8 and 3G9 with cysteine, acetylcysteine and several mercapturic acids were assessed. Supernatant 3G9 cross-reacted with S-BMA and S-PMA-methy ester when they were present in 45 to 88 fold molar excess (Figure 3.6). IC50 values obtained with 6B2, 3G9 and 6B8 in cross-reactivity studies are summarised in table 3.8.

TABLE 3.8. CROSS REACTIVITY STUDIES WITH 6B2, 3G9 AND 6B8

INHIBITOR	IC50 VALUE (mM)		
	6B2	3G9	6B8
S-PMA	0.25	0.002	0.01
CYSTEINE	-	-	-
ACETYLCYSTEINE	-	-	-
2-HEMA	-	-	-
HYDROXYPROPYL-MA	-	-	-
ALLYL-MA	-	-	0.34
S-BMA	-	0.14	0.41
S-PMA-METHYLESTER	-	0.24	0.07

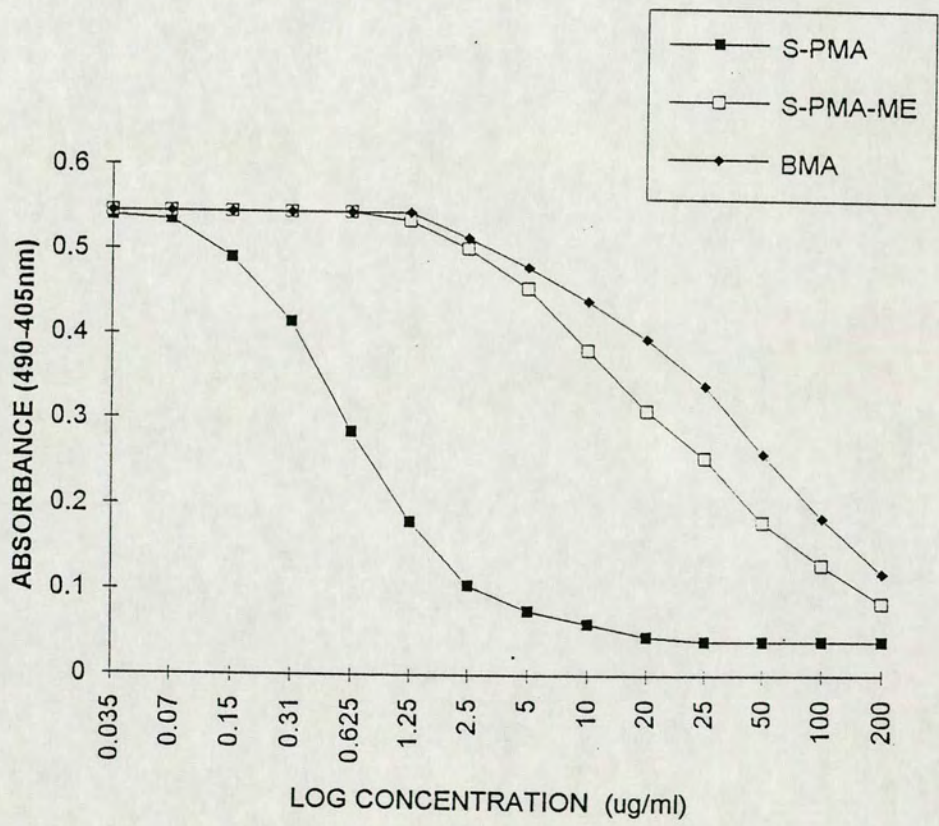


Figure 3.6 Cross-reactivity studies with antibody 3G9

S-(2-HYDROXYETHYL)-N-ACETYLCYSTEINE

3.20.1 Fusions 3-17 (in-vivo immunisation)

All the fusions were performed successfully, and wells which contained one or more viable colonies were found on all the fusion plates (Table 3.9). Fusion efficiencies, defined as the number of wells containing hybridomas as a percentage of the total number of wells, ranged from 38% to 100%.

TABLE 3.9 FUSION RESULTS FOR MICE IMMUNISED WITH 2-HEMA CONJUGATES

IMMUNOGEN	MOUSE	WELLS PLUS HYBRIDS	POSITIVE IN SCREEN	POSITIVE IN INHIBITION STUDY
KLH-ABS-HEMA	1	276	56	0
	2	210	51	0
	3	288	44	0
GEL-BALA-HEMA	1	138	4	0
	2	252	9	0
KLH-7C-HEMA	1	360	110	6
	2	240	3	0
GEL-7C-HEMA	1	324	30	0
	2	342	114	10
MSA-7C-HEMA	1	252	12	1
	2	204	10	0
	3	90	0	0
	4	140	0	0
	5	360	6	2
	6	360	55	1

The number of positive wells varied considerably between fusions. Two fusions (MSA-7C-HEMA immunised mice 3 and 4) contained no positive supernatants in the screening assay. Up to 33% of the wells

which contained clones were positive in the ELISA. There was considerable variation in the colour intensity of the positive wells. In general the absorbance was low and wells were recorded as positive with an O.D. of two times the background. Approximately 2% of the positive wells in the ELISA contained no viable hybridoma cells.

Binding of free hapten by antibody was only detected and confirmed in a repeat assay for twenty supernatants (4% of the positive wells in the screening assay). The percentage inhibition varied between the supernatants, but was generally low. With 100ug/ml 2-HEMA standard inhibition ranged from 20% to 74% (Table 3.10).

TABLE 3.10 INHIBITION WITH 2-HEMA

IMMUNOGEN	MOUSE	WELL	% INHIBITION
KLH-7C-HEMA	1	1C3	55
		1G10	25
		5B3	26
		5D6	24
		6C2	66
		6F9	42
GEL-7C-HEMA	2	1F2	60
		2B3	74
		2F6	63
		2G11	37
		4B4	47
		4B9	37
		4E6	70
		4E9	31
		5E8	45
6B11	28		
MSA-7C-HEMA	1	5D2	38
	5	1C11	21
		2B5	28
	6	1B5	27

Positive wells were also screened by RIA. In assays where separation of bound and free label was effected with 5% dextran coated charcoal total counts were 10,000dpm, and the background after separation was 1414dpm (SD=69.8, n=10). In assays separated by saturated ammonium sulphate solution less label was added to the assay. The totals were 1978dpm and the background was 25.5dpm (SD=5.08, n=15). No positive wells were detected by RIA.

The twenty positive wells were cloned and expanded as described in methods. Monoclonality was not achieved despite exhaustive attempts to isolate the hybridoma cells.

3.20.2 Fusions 18-20 (in-vitro immunisations)

STUDY 1:-

Spleen cells from the GEL-7C-HEMA immunised mouse were successfully stimulated in-vitro. Cultured cells were examined daily microscopically. On day 2, expanded lymphocytes, or blast cells, were observed (Figure 3.7). The blast cells were approximately ten times larger than normal lymphocytes. The blast cells divided in culture, days 3-4, forming colonies (Figures 3.8 and 3.9). On day 5 the cultured cells were fused as described in materials.

The fusion was performed successfully, and the plating efficiency was greater than 90%. On day 12, supernatants were screened against plates coated with BSA-7C-HEMA. 60 wells (18% of the wells containing clones) contained antibody which bound the coating conjugate. Only one supernatant contained antibody which bound free mercapturic acid (58% percent inhibition with 100ug/ml 2-HEMA). The antibody was

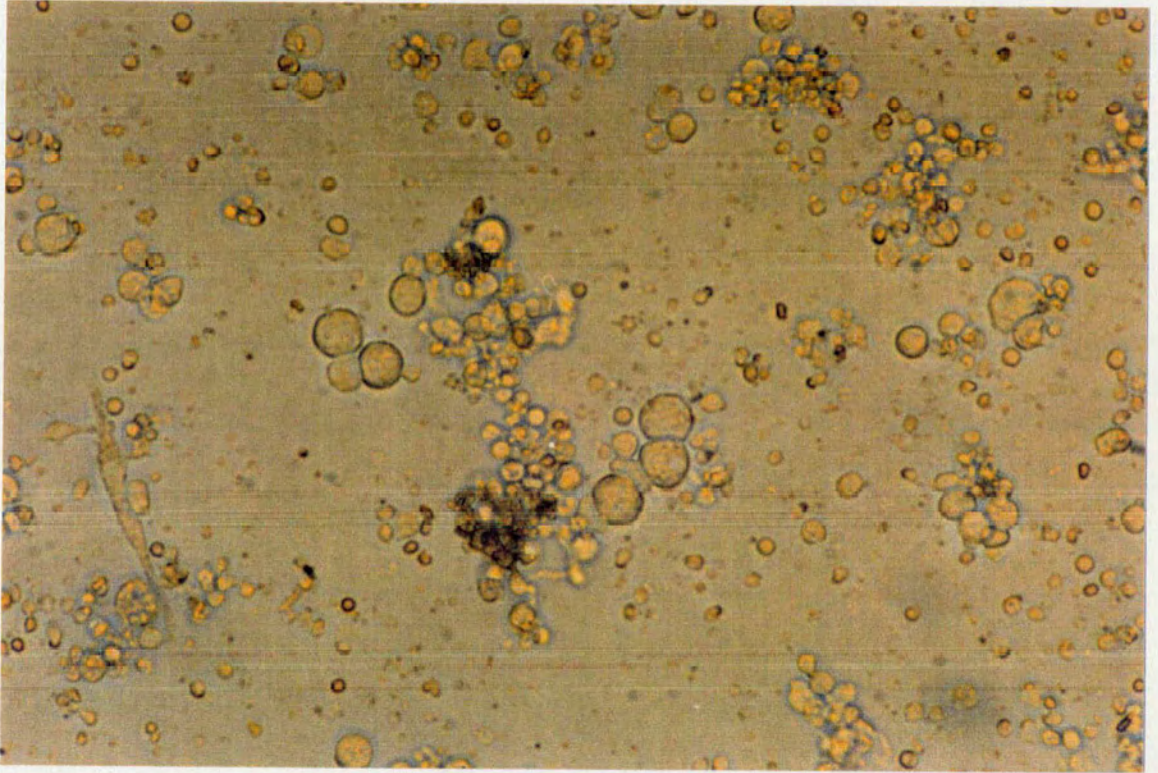


Figure 3.7 Blown cells formed after 2 days in in-vitro culture in the presence of conjugate.

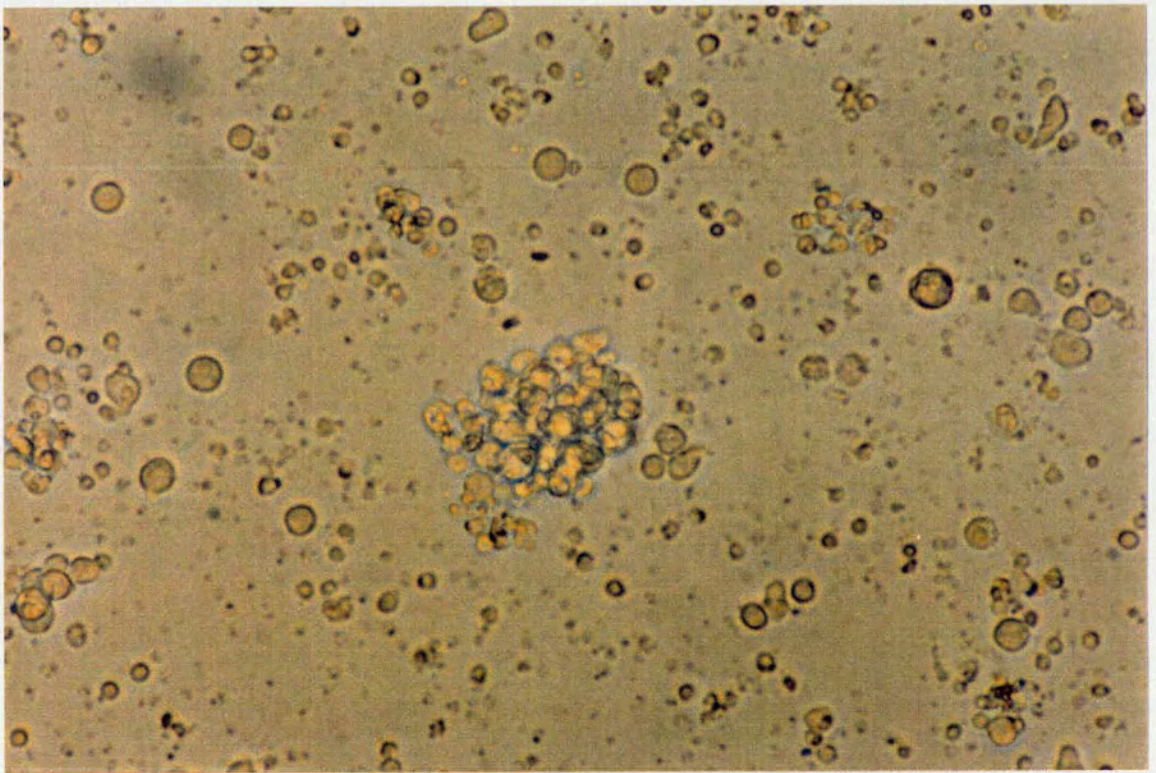
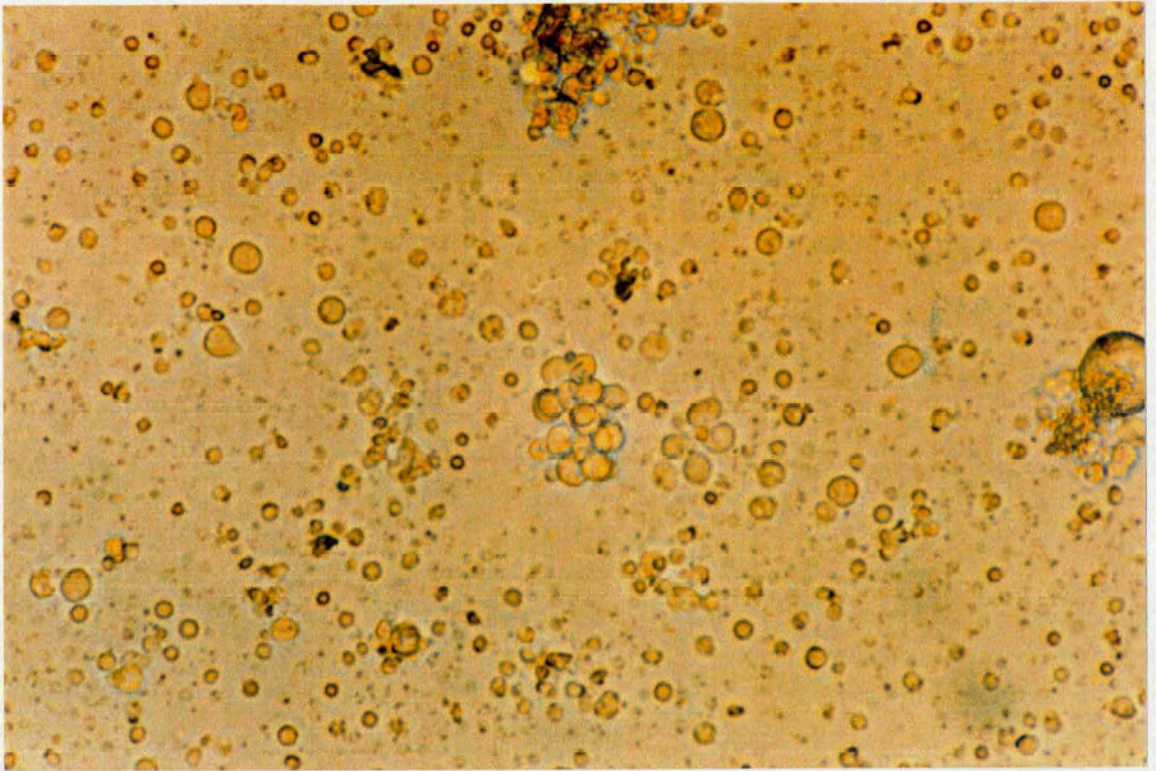


Figure 3.8 and 3.9 Blast cells observed after 5 days in in-vitro culture in the presence of conjugate.

specific for 2-HEMA. The clone plates were frozen at -180°C .

STUDY 2:-

The number of viable cells on the day of fusion varied considerably when lymphocytes were primed in-vitro, in-vivo and immunised in-vitro (Table 3.11).

TABLE 3.11 A COMPARISON OF IN-VIVO AND IN-VITRO IMMUNISED MOUSE FUSIONS

IMMUNISATION PROTOCOL	NOS. VIABLE CELLS	WELLS CONTAINING HYBRIDOMAS	NOS. POSITIVE WELLS IN SCREEN	NOS. POSITIVE WELLS IN INHIBITION STUDY
IN-VIVO BOOST	1×10^8	240 (67%)	110	6
IN-VITRO BOOST	1.2×10^6	70 (39%)	61	2
IN-VITRO IMM.	1×10^4	28 (47%)	18	0

The results of the in-vivo boosted fusion have been presented (3.20.1). Fusions with both in-vitro boosted and primary immunised spleen cells were successful. On screening, the number of wells containing one or more clones was determined microscopically. More hybridoma clones were observed with the in-vitro boosted spleen cell fusion (70 in-vitro to 28 in-vivo). Supernatants from both fusions contained antibodies which bound coating conjugate in the screening assay.

The number of positive wells in the screening assay was greater after in-vitro boosting (61 compared to 18). Only the in-vitro

boosted lymphocytes gave rise to antibodies which bound free hapten. Two wells (6D8 and 6F8) demonstrated inhibition (69% and 97% respectively) in the inhibition study. Neither were positive in the RIA. One well (6B2) gave a positive result in the RIA but did not show inhibition in the ELISA. The positive well contained sixteen times more label than the background (positive well = 300dpm, background = 26dpm). All three wells contained rapidly dividing cells and were cloned immediately. No antibody activity was detected in subsequent screening assays.

3.21 MSA-ACETCYSTEINE IMMUNISED MICE

Spleen cells from mice 1, 2 and 4, immunised with acetylcysteine conjugated to MSA, were successfully fused with the Sp2 myeloma cell line. On the day of screening 90%-100% of the wells per fusion contained one or more viable clones. Supernatants were screened against plates coated with 0.1ug/ml of BSA-acetylcysteine conjugate. Fourteen, twenty and forty two positive wells were detected in each fusion respectively (table 3.12).

There was considerable variation in the colour intensity of the wells. Wells that were recorded as positive in the screening assay had an O.D. greater than two times the background. All the positive wells contained multiple small and large clones.

Seventeen supernatants bound free hapten (2-HEMA and S-PMA) in the inhibition studies. In the inhibition study for mouse 1 only two of the positive supernatants from the screening assay had O.D. values greater than twice the background. The absorbance values were low in

general. Results of the inhibition study (supernatants with OD more two times background) are shown in table 3.13.

TABLE 3.12 SCREENING RESULTS FOR MSA-ACETYLCYSTEINE FUSIONS 1-3

FUSION	PLATE	NOS. POSITIVE WELLS IN SCREEN	NOS. POSITIVE WELLS IN INHIBITION STUDY
MOUSE 1	1	1	1
	2	1	1
	3	8	5
	4	4	2
	5	0	0
	6	0	0
MOUSE 2	1	2	2
	2	7	1
	3	2	0
	4	4	1
	5	3	2
	6	2	0
MOUSE 4	1	9	0
	2	3	0
	3	6	1
	4	2	1
	5	7	0
	6	15	0

Eight positive wells (mouse 1: 1D3, 2B10; mouse 2: 1B9, 1G9, 2F7, 5G11 and mouse 4: 3C5, 4F8) were cloned and expanded as described in the methods. On the day of screening, all the plates contained viable hybridoma clones. Only 3 clone plates contained wells which gave a positive result in the screening assay. For all the positive wells the absorbance readings at the end of the assay were relatively weak.

TABLE 3.13 INHIBITION RESULTS FOR MSA-ACETYLCYSTEINE FUSIONS 1-3

FUSION	WELL	% INHIBITION	
		S-HEMA	S-PMA
MOUSE 1	1D3	75	80
	2B10	17	55
MOUSE 2	1B9	83	83
	1G9	70	77
	2F7	38	73
	4D3	42	76
	5E7	67	69
	5G11	68	72
MOUSE 3	3C5	22	23
	4F8	11	66

Clone plates 1B9 and 2F7 contained 12 and 5 positive wells respectively. All the positive wells contained several clones when examined microscopically. No positive wells were observed after a second round of cloning.

Clone plate 3C5 gave 32 positive wells in the screening assay. Three of the positive wells appeared monoclonal when examined microscopically. Monoclonality was confirmed for well 3C5D9.

Antibody titre was 1:4 for the harvested supernatant. The relative affinity of the antibody for 2-HEMA, S-PMA and acetylcysteine was determined from calibration plots (Figure 3.10). IC50 values with 2-HEMA, S-PMA and acetyl cysteine were 0.15, 0.24 and greater than 0.48 mM respectively.

3C5D9 did not bind C¹⁴ 2-HEMA in the RIA.

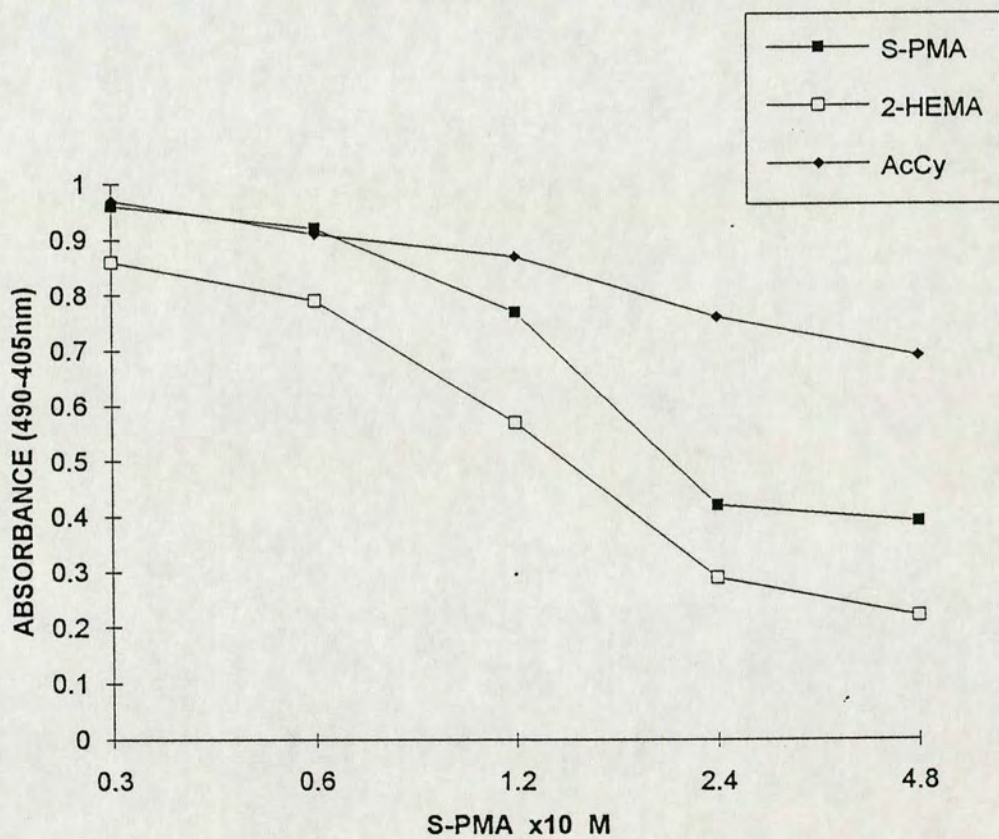


Figure 3.10 2-HEMA, S-PMA and acetylcysteine calibration plots developed antibody 3C5

DISCUSSION

With a carefully designed hapten-protein conjugate and a judicious screening strategy, it was possible to generate and detect antibodies which bind S-PMA. Using hybridoma technology three monoclonal cell lines have been established which secrete antibody with relatively high affinity and specificity for S-PMA. The cell lines have been cultured in stationary flasks and the media harvested. 2-HEMA and acetylcysteine conjugate immunised mice have also been fused. Antibodies which recognise free mercapturic acid were detected but the affinities were too low for practical application. The results of the fusions are discussed below.

Six mice were immunised with the protein conjugates KLH and HSA-PMA. Two KLH-conjugate immunised mice (3 and 1) were selected for fusion. Mice 3 and 1 were selected because these sera gave the highest titres (1:25000) and greatest inhibitions (26% and 22% respectively) in the screening assays (see chapter 2).

Both fusions were performed successfully and plating efficiencies were high (average 93%, range 60%-100%. tables 3.1 and 3.3). Supernatants with a high absorbance in the screening assay were selected for competitive inhibition studies. These supernatants should contain a greater proportion of hybridoma cells secreting antibody which binds S-PMA-conjugate, or hybridomas secreting antibody of high affinity.

In the inhibition studies 82% of the positive wells bound free hapten. In fusion 1, well 6B2 demonstrated the greatest inhibition (79%, Table 3.2). In fusion 2, six wells demonstrated almost

complete inhibition in the binding assay (Table 3.4). Wells 3G9 and 6B8 demonstrated the highest affinity for free S-PMA (Table 3.5). Wells 6B2, 3G9 and 6B8 were therefore selected for further study and cloned. Monoclonality was confirmed for all three cell lines.

Cell line 3G9 had the greatest relative affinity for S-PMA (Table 3.6). The limit of detection of a calibration plot developed with 3G9 was 0.04ug/ml of S-PMA (Figure 3.4). Calibration curves prepared with 3G9 were 250 and 5 fold more sensitive than those prepared with 6B2 and 6B8 (limit of detection 10 and 0.2ug/ml respectively).

The aim of this study was to produce an anti-PMA monoclonal antibody and to develop an immunoaffinity column to enrich S-PMA from the urine of benzene exposed workers. The performance of the antibodies in the presence of urine was therefore assessed. Binding between antibody 3G9 and S-PMA was not affected by the presence of urine (Figure 3.5). However, with supernatants 6B2 and 6B8 antibody-antigen binding was affected by the presence of urine, leading to a 40 fold loss in assay sensitivity (table 3.7).

Antibody cross-reactivity was assessed against a number of similar analogues (Table 3.8). No cross-reactivity was observed with 6B2. However, 6B2 demonstrated a relatively low affinity for S-PMA. Supernatants 3G9 and 6B8 showed no cross-reactivity with cysteine, acetylcysteine and 2-HEMA. Both supernatants showed some cross-reactivity with a large excess of S-PMA-methylester and S-BMA. 3G9 demonstrated the greatest specificity, and cross-reactivity with S-PMA-methylester and S-BMA was only observed when they were present in a 120 and 70 fold molar excess respectively.

Cross-reactivity studies indicate that both the phenyl and carboxylic acid groups of the mercapturic acid appear to be important in antibody-antigen binding. Both probably form the epitope bound by the antibody's active site. Confirming the importance of the carboxylic acid in antibody-antigen interaction postulated in the design of the hapten-protein conjugate (chapter 2).

Supernatant from cell line 3G9 demonstrated the greatest relative affinity for S-PMA. The antibody was specific for S-PMA and the performance was not affected by the presence of urine. Supernatant 3G9 was therefore selected for the development of an S-PMA immunoaffinity column. (see chapter 4).

In contrast to S-PMA the production of an antibody which binds 2-HEMA with high affinity has proved to be very difficult. In total, fifteen mice were boosted in-vitro and the spleen cells fused successfully. In general plating efficiencies were high (up to 100%), but for three mice the plating efficiencies were less than 50% (Gel- β ala-HEMA mouse 1, MSA-7C-HEMA mice 3 and 4, Table 9). Sera from these mice had low antibody titres (less than 1:150, Figure 2.10) when screened after the second immunisation. Poor stimulation of the lymphocytes and a lack of antigen induced proliferation may account for the relatively low numbers of hybridoma cells. Two of these fusions (MSA-7C-HEMA mice 3 and 4) gave no positive wells in the screening assay.

All the remaining fusions contained wells which gave a positive result in the screening assay. In general, the number of positive

wells was low (1%-33% of the wells containing viable clones) and the colour development at the end of the screening assay was weak. This contrasted strongly with the results of the S-PMA fusions above. 2% of the positive wells did not appear to contain any viable cells when examined microscopically. These wells may have contained viable cells which subsequently died. Alternatively, absorbance values selected as positive in the screening assay (two times background because of poor colour development) were too low giving rise to false positives.

In general the number of positive wells per fusion increased as the number of wells containing clones increased. There was no obvious difference between fusions where lymphocytes were stimulated with KLH, gelatin and MSA-2-HEMA conjugates. In the ELISA, mice immunised with the Gel- β -ala-2-HEMA conjugate contained relatively few positive clones. However, this fusion was screened against a conjugate with a different spacer arm to the immunogen (BSA-ABS-HEMA) and probably represents the response that recognises the hapten and not a hapten-spacer complex.

Binding studies were performed with a high concentration of 2-HEMA (100ug/ml, 4.2×10^{-7} moles/ml) but the inhibition was generally low (average 42%, range 21%-70%) indicating that the antibodies were of a relatively low affinity. The failure to generate high affinity antibodies to low molecular weight compounds has been reported by Chappey (1994).

The limitations of the ELISA for screening low molecular weight compounds are discussed in chapter 2. In attempts to overcome these

limitations, positive wells were also screened by RIA. Supernatants were screened against 3×10^{-11} or 1.5×10^{-10} moles/ml 2-HEMA. No positive results were obtained in the RIA, confirming all the antibodies which bound hapten in the ELISA were of low affinity.

To improve the efficiency of specific hybridoma production in-vitro boosting of in-vivo primed cells was examined. Initially, cells were cultured in serum free protein free medium to minimise the non-specific activation of lymphocytes by serum proteins. However it was found that low concentrations of foetal calf serum (5% Myclone Plus) were necessary to support cell growth. Under the culture conditions employed lymphocytes could be cultured for five days in the presence of immunogen. After five days the number of viable cells declined rapidly, resulting in poor fusion efficiencies.

Two mice were boosted in-vitro with two different conjugates (gelatin and KLH-7C-HEMA). Similar results were obtained with both fusions. In the presence of immunogen it was observed that a number of lymphocytes increased in size (Figure 3.7). It was presumed that these were antigen stimulated cells, or blast cells, described by Borrenboeck (1986). These cells divided to form colonies (Figure 3.8 and 3.9) which were either anchored to the tissue culture flasks or floating free in the culture media.

Lymphocytes immunised in-vitro were fused successfully. Plating efficiencies (up to 90%) compared with those obtained using in-vivo boosted spleen cells. Antibodies which bound the conjugated hapten were detected in the screening assay. The assay results compared with the in-vivo boosted mice results.

One supernatant containing antibody which bound free 2-HEMA was generated with the gelatin conjugate. This supernatant was only screened by ELISA since there was insufficient supernatant to screen by RIA. The plates from this fusion were frozen before cloning and were unsuccessfully thawed at a later date. Two supernatants which bound free 2-HEMA in the ELISA (Table 3.11) were generated with the KLH conjugate. However these antibodies were of low affinity and did not bind the labelled hapten in the RIA. One well (6B2) gave a positive result in the RIA but did not show any inhibition in the ELISA. This can be explained if the antibody had a high affinity for free 2-HEMA (positive in RIA) but a higher affinity for hapten coupled to the carrier protein (negative in ELISA).

Finally, mice were also immunised with MSA-acetylcysteine conjugate. The results of the fusions (Table 3.12) were comparable to those described above. The number of positive wells (approximately 4%-12% of the wells containing clones) compared with results obtained with 2-HEMA stimulated spleen cells. However, the number of cells which bound free hapten in the ELISA was greater than expected (5%-64% of the positive wells). All of these antibodies were of low affinity as no binding was observed in the RIA.

Supernatants were screened against both 2-HEMA and S-PMA by ELISA. The inhibitors were of equimolar concentration. However, inhibition was generally greater with the S-PMA inhibitor showing that this mercapturic acid was recognised by the antibodies with greater affinity (Table 3.13). It was also observed that some antibodies recognised non-modified MSA in the screening assay. That is, some antibodies generated to the hapten-MSA complex also cross-reacted

with native MSA. Autoantibodies were induced.

It is often difficult to see how such antibodies may arise (Roitt 1980). In the body mechanisms exist to prevent the recognition of "self" components as antigens. However these can break down leading to antibodies which recognise "self" - autoantibodies. Autoantibodies lead to a spectrum of autoimmune disease, such as organ-specific Hashimoto's disease of the thyroid, or the non-organ specific disease Systemic Lupus Erythematosus (SLE).

Serum proteins are exposed to many chemical insults every day. The induction of antibodies to modified proteins which cross react with native protein has been demonstrated. It is easy to see how devastating this could be on the body when the catalytic activity of some antibodies are taken into consideration (Schultz 1988).

Despite exhaustive attempts to clone the cells (from both 2-HEMA and acetylcysteine-conjugate immunised mice) which showed inhibition, monoclonality was only obtained with one cell line (MSA-acetylcysteine immunised mouse 3 well 3C5). There are a number of possible explanations for this. After fusing, dividing hybrids may lose chromosomes to obtain a more stable genotype. There is a tendency for chromosome loss to occur after the initial cloning and more than 50% of the clones can be lost at the first round cloning. With successive rounds of cloning cell stability increases (Edwards 1981).

Furthermore, due to the low number of positive wells in the screening assays many weakly positive wells were cloned. It was

observed that all these wells contained a number of large and small viable hybrid colonies and therefore, only a low proportion of cells may have been secreting antibody of interest. The few specific hybridoma cells could easily be overgrown by a large number of rapidly growing non-secretors.

Finally, some cell lines have inherently poor cloning efficiencies, and may not have grown at low dilution even in the presence of a layer of thymocyte feeder cells.

From the above it can be seen that the positive selection of hapten specific hybridomas would greatly facilitate the cloning process. Parkes et al (1979) identified antigen specific hybridomas with fluorescent microspheres and cloned using a fluorescent activated cell sorter (FACS). Cloning with a FACS is expensive and the method is unsuitable for routine use. Magnetic beads have been used for the positive selection of cell populations by a number of authors, for example T and B lymphocytes (Lea et al 1985) and malignant cells from bone marrow (Treleaven et al 1984, Kvalheim et al 1987). Cells were incubated with magnetic beads coated with antibody to a specific surface antigen, rosettes of antigen bearing cells were formed which could be separated from the mixture with a powerful magnet. Horton et al (1989) described an adaptation of the method selecting antigen specific hybridoma cells using magnetic beads coated with hapten. Selected cells were cloned by limiting dilution with the beads still bound to their surface. He found a greater number (250%) of cell lines could be established in 50% of the normal time. The interaction of lymphocytes with magnetic polymer

particles has also been studied by Ossendorp et al (1989) and Pilling et al (1989). All three authors describe similar optimised conditions and their results confirm that coated beads can be used for the efficient selection of high affinity B cell hybridomas.

In order to improve cloning efficiency the use of antigen coated magnetic beads to select lymphocytes bearing specific antibody on their surface was investigated. The spacer arm B-alanine was coupled to tosyl activated magnetic beads (Dyna). 2-hydroxyethylcysteine was then coupled via its amino group to the β -alanine carboxylic bead using N-hydroxysuccinimide and carbodiimide. Cells and 2-HEMA coupled beads were mixed and incubated. The beads were separated using a selenium magnet. After separation cells were counted (during counting several beads were observed on the surface of some of the cells) and cloned by limiting dilution. When the clone plates were screened the number of positive wells was increased 2-3 fold. However it appeared cells secreting antibody with a high affinity for 2-HEMA plus spacer were selected since none of the supernatants demonstrated inhibition in the binding studies. Horton et al (1989) reported that the use of antigen coated beads selected cells that secreted antibody of high affinity ($<10^7$ l/mole), and this method may be unsuitable for cloning cells bearing low affinity antibody.

This study demonstrates that the number of B cells secreting high affinity antibody form a very small percentage of the total response. Cell fusion is inefficient, the genetic information of only about 1:10000 lymphocytes is fixed in hybrid cells. In addition, it is not always possible to isolate these cells during cloning. Molecular biology techniques are been increasingly used to

generate monoclonal antibodies (for a review see Owens and Young 1994). With recombinant antibody technology the entire gene repertoire can be saved and expressed. Recombinant technology should increase the chances of generating high affinity antibodies to 2-HEMA. In addition, with mutagenesis it may be possible to alter the genetic framework of a low affinity 2-HEMA antibody to give an antibody with high affinity for 2-HEMA.

CHAPTER FOUR

IMMUNOENRICHMENT OF URINARY S-PHENYLMERCAPTURIC
ACID

INTRODUCTION

Benzene is an important industrial chemical, and the current occupational exposure limits for benzene (8 hour Time Weighted Average or TWA) in the United Kingdom (Maximum Exposure Limit or MEL), European Union and United States (Permissible Exposure Limit or PEL) are 5, 1, and 1ppm respectively. In order to comply with a limit value of 1ppm, 8 hour TWA exposures must be well below this value most of the time. Methods sensitive enough to measure below 1ppm are therefore needed.

4.1 ANALYTICAL METHODS FOR THE DETERMINATION OF URINARY S-PMA

Urinary S-PMA is a sensitive and specific marker of benzene exposure (chapter 1), and several techniques have been described for the quantitative analysis of S-PMA. These include amino acid analysis, HPLC and GC (fig. 4.1). At present only GC/MS determination of S-PMA possesses the required sensitivity for biomonitoring occupational exposure to benzene.

4.2 GC/MS DETERMINATION OF URINARY S-PMA

Van Sittert et al (1993) determined S-phenylmercapturic acid in human urine using a modification of the method described by Stommel et al (1989). Briefly, the urine is acidified and the mercapturic acid extracted into ethyl acetate. Following centrifugation the ethyl acetate is removed and evaporated to dryness under a gentle stream of nitrogen in a water bath at 45⁰C. The residue is resuspended in methylating agent (methanol/1.25M HCl) After two hours

FIG.4.1 Analytical Techniques and Detection Limits for the measurement S-phenylmercapturic acid in urine.

METHOD OF SEPARATION	METHOD OF DETECTION	LIMIT OF DETECTION	REFERENCE
Partition analysis	Amino acid	2nmol/ml	Stommel (1989)
HPLC	UV	6nmol/ml	Jongeneelen (1987)
HPLC	Fluorescence	1nmol/ml	Norpath (1991)
HPLC	ECD	20pmol/ml	Norpath (1991)
GC	MS-SIM	50pmol/ml	Stommel (1989)
GC	MS-SIM	10pmol/ml	Norpath (1991)
GC	MS-SIM	4pmol/ml	Van Sittert (1993)

at room temperature the methylating agent is evaporated and the sample dried as above. The residue is resuspended in dicloromethane for analysis by GC/MS.

The methylester of S-PMA is analysed by mass spectrometry in an electron impact mode (EI) with selective ion monitoring (SIM). Under these conditions the most abundant ion has an m/z of 194.

In order to avoid misinterpretation of the spectra due to minor interferences by other components, a "qualifier ion" (another fragment of lesser abundance or specificity than the "selected ion") is monitored along side the "selected ion". It should be checked that the "qualifier ion" has the same retention time in the chromatographic system as the "selected ion", and that its abundancy

in comparison with the "selected ion" matches its relative abundance in the mass spectrum of the pure compound. The methylester ion (m/z 253) was chosen as the "qualifier ion".

Van Sittert et al (1993) used the GC/MS method described above to measure levels of urinary S-PMA in three industrial settings where benzene exposure can occur: oil refineries, chemical manufacturing and natural gas production plants. They confirmed that S-PMA is a sensitive and specific marker of benzene exposure, allowing exposures down to 0.3ppm 8 hour TWA to be determined. Depending upon matrix effects the limit of detection of the assay was 1-5ug/l when S-benzylmercapturic acid (S-BMA) was used as an internal standard. In highly concentrated urine samples the S-PMA peak could appear a few milliseconds later in the chromatograph compared to the internal standard. Because of this, the S-PMA peaks from concentrated samples, with S-PMA values of >5ug/l, could not always be attributed with certainty. In addition, S-BMA is found in the urine of toluene exposed workers. Toluene and benzene are commonly used co-solvents and co-exposure may occur. The presence of S-BMA in the urine of toluene exposed workers interferes with the S-BMA internal standard.

Recently, Boogaard and Van Sittert (1995) have described the use of S-(pentadeuterophenyl) mercapturic acid (d5-S-PMA) as an internal standard. D5-S-PMA was synthesised from d5-aniline and N-acetylcysteine according to the Gattemann reaction. Using d5-S-PMA a detection limit of 1ug/l could be guaranteed. Using d5-S-PMA background levels of S-PMA could be detected in non-exposed populations.

A limitation of the S-PMA method is that determination by GC/MS requires a complex work-up prior to assay and the sensitivity of the procedure is limited by the purity of the sample. The development of an effective single step immunoextraction procedure to replace this may both facilitate and improve the sensitivity of the quantitative MS analysis of S-PMA.

In addition GC/MS analysis restricts this method to use in laboratories with sophisticated analytical equipment. The use of an immunoaffinity column to enrich S-PMA may permit the analysis of S-PMA by HPLC which at present lacks the sensitivity for application in biomonitoring.

4.3 IMMUNOAFFINITY CHROMATOGRAPHY

Affinity chromatography is a method of fractionation in which the molecule to be purified is specifically and reversibly bound by a complementary binding molecule (the ligand) attached to a solid support (the matrix).

In immunoaffinity chromatography either an antibody or an antigen can be immobilised to form the adsorbent (immuno-adsorbent or immunosorbent). With the development of monoclonal antibody technology the use of antibodies as ligands has become widespread. Using monoclonal antibodies, adsorbents of defined specificity and affinity can be prepared. With antibody adsorbents, rapid separations (which would be difficult and time-consuming by other techniques) and purification factors of several thousand are

possible. Immunoaffinity chromatography has been reviewed by Goding (1986) and Jack and Wade (1987).

4.4 ANTIBODY PURIFICATION

Protein A and protein G are two proteins of staphylococcus origin. Protein A binds immunoglobulins IgG (IgG₁ poorly) and IgM, whilst protein G binds IgG only. Protein G binds immunoglobulins with a greater avidity than protein A. Supernatants can be loaded directly on to protein A and G columns. Antibody can be eluted with pH 4 buffer from protein A. Antibodies are generally stable under these conditions. To elute antibodies from protein G harsher elution conditions are needed. Antibodies are normally eluted at pH 2 but prolonged exposure at pH 2 is detrimental to the immunoglobulins and the samples must be immediately neutralised. For optimum performance the sample load should be approximately half of the column capacity, minimum flow rates allowing for maximum interaction.

4.5 PREPARATION OF AN IMMUNOAFFINITY COLUMN WITH 3G9

CHOICE OF SOLID PHASE

When preparing an immunoabsorbent the first decision is the choice of solid support. A range of solid supports are available and these have been reviewed by Narayanan and Crane (1990). The ideal matrix exhibits high specificity, contains no hydrophobic binding sites, possesses good chemical stability, excellent mechanical rigidity, high binding capacity, has a low cost and good reproducibility is yet to be synthesized (Narayanan and Crane 1990). Beaded agarose comes closest to these ideals. Beaded agarose has high porosity,

hydrophilicity and chemical stability. Sepharose is a bead formed agarose gel. It possesses an open pore structure which is available even to large molecules. The exclusion limit of sepharose 4B in gel filtration studies is MW 20×10^6 (Pharmacia). It can tolerate undiluted ethanol, methanol, butanol, acetone, dioxane, 80% v/v aq, pyridine and 50% DMF. Its surface is relatively inert, and contains hydroxyl groups which can be easily activated. However, there are disadvantages. It cannot withstand high pressure which limits the column flow rate, it has a relatively low binding capacity and is susceptible to microbial degradation. Freezing causes irreversible changes in structure.

COUPLING CHEMISTRY

Many different coupling methods are available (for a review see Narayanan and Crane, 1990). The use of CNBr activated sepharose to couple antibodies is very popular. Preparation of CNBr activated sepharose is simple and inexpensive, but uses a volatile and toxic chemical (cyanogen bromide). Pre-activated agarose is available as a stable lyophilized powder. CNBr activation increases the number of crosslinks between the polysaccharide side chains improving the stability of the agarose. After activation and coupling the solid support can withstand high concentrations of salt, urea, guanidine-HCl, SDS, deoxycholate and Triton X.

4.16 CNBR ACTIVATED SEPHAROSE

Antibody coupling is achieved by the nucleophilic attack of the epsilon amino group of lysine on CNBr activated sepharose forming a stable isourea bond. Maximum binding occurs under slightly alkaline

conditions when the amine group is unprotonated. Minor competing reactions lead to the formation of carbamate and cyclic imidocarbamate (fig. 4.2). Suitable buffers for coupling are PBS, 0.1M sodium borate and 0.1M sodium carbonate pH 7.5-8.5. The presence of Tris, ammonium and azide ions will inhibit coupling. Protein binding capacity varies with the degree of activation, pH and antibody. CNBr activated sepharose 4B has a binding capacity of 10-15mg of protein per ml of wet gel.

The total amount of protein reacted with activated gel is important. Coupling efficiencies of 100% are possible but generally undesirable for maximum antibody activity. Optimum results appear to be achieved when 80%-90% of the antibody is coupled to activated gel (Pfeiffer et al 1992). This seems to be due to the number of points of attachment. Over-attachment leads to a loss of tertiary structure necessary for antigen-antibody interactions. Westen and Scuver (1977) found 3-4mg of IgG coupled per gram of sepharose give maximum binding capacities. At higher antibody densities binding capacity was diminished, possibly due to steric hindrance and the inaccessibility of the antibody in the pores of the support.

After coupling, unreacted cyanate or imidocarbamate groups (fig. 4.2) are blocked. Blocking can be performed with glycine, ammonium hydroxide or ethanolamine. Glycine and ethanolamine introduce other ionically charged groups into the matrix. Non-specifically adsorbed protein can be removed by alternate washes with low and high pH buffers.

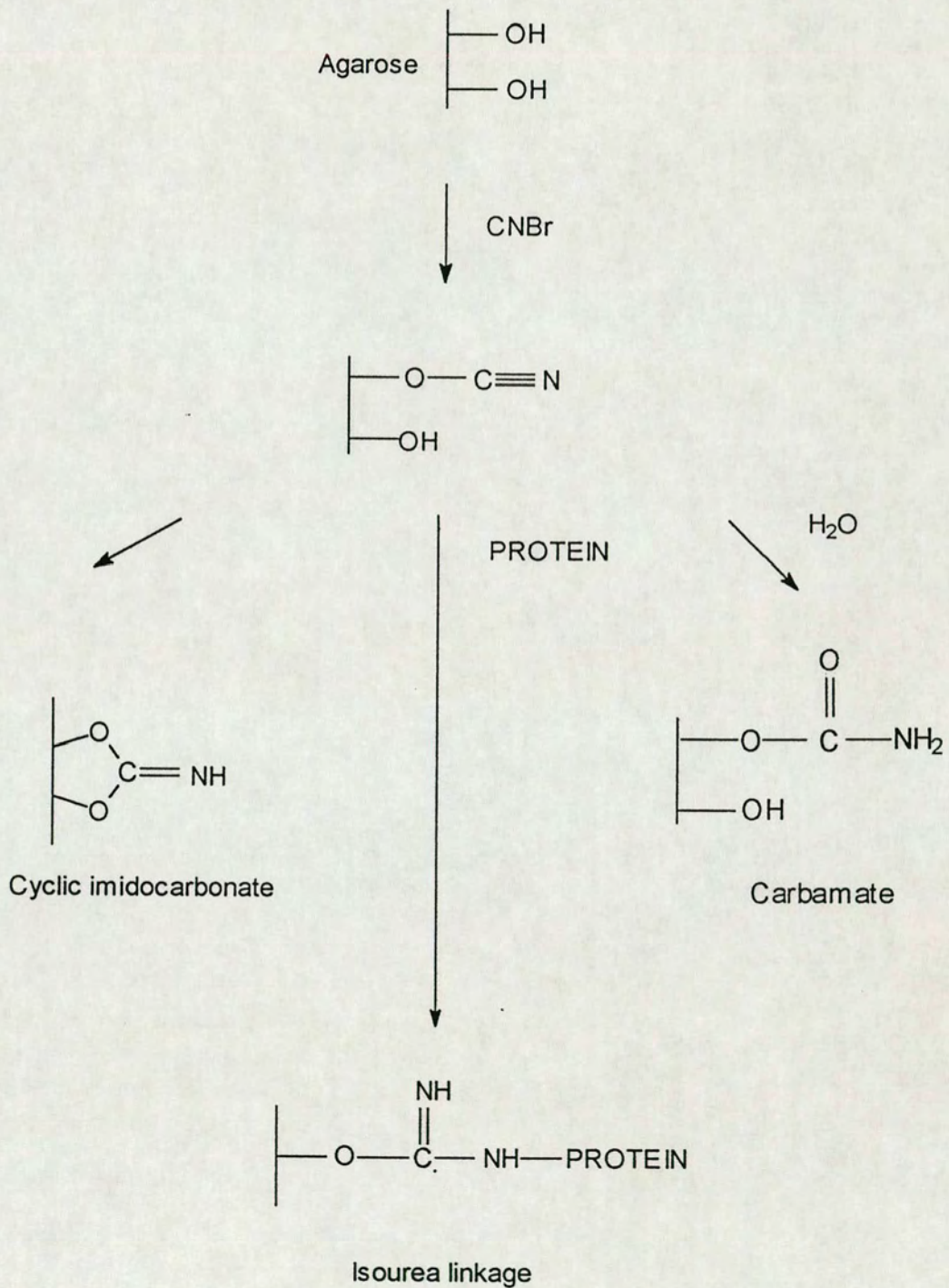


Figure 4.2 CNBr Activated Sepharose - antibody coupling chemistry

4.7 USE OF IMMUNOAFFINITY CHROMATOGRAPHY COLUMNS

Individual monoclonal antibodies possess different chemical and physical properties. Optimised chromatography conditions will therefore vary between immunoaffinity columns prepared with different antibodies.

For binding, the antigen should be soluble and free of aggregated material or debris which would block the column. Insoluble impurities can be removed (pre-column clarification) by filtration or centrifugation. With small columns a flow rate of 1-2ml/min is customary. Cross-linked matrices give better flow rates, compress less and maintain better flow characteristics. With solid supports of a porous nature diffusion within the structure and antibody accessibility may limit the flow rates which can be achieved.

Column efficiency, or performance capability in terms of purity and yield, will be determined by the affinity constant of the column (or antibody). Affinity constants can be determined by equilibrium experiments (Scatchard analysis). Both temperature and pH can have an effect on the affinity constant (Eveleigh and Levy 1977). High (37°C) and low (4°C) temperatures lead to a decrease and increase in the affinity constant respectively. The effects of temperature (a factor of 2 in the above range) is negligible compared to the effect of pH. Affinity constants are greatest at pH 6-8 and fall rapidly outside these two values. Binding was therefore performed at about pH 7.

After binding, columns are washed, usually with 5-20 column volumes of binding buffer. Care should be taken with low affinity antibodies that excess washing does not elute the antigen. Non-specific binding is a property of the matrix and individual molecules. Electrostatic effects are largest at low salt concentrations. They can be pH sensitive. Hydrophobic interactions can also occur. These tend to occur with hydrophobic spacer arms. Hydrophobic effects are increased at high salt concentrations. The effect of salt and pH vary with different antibodies.

Elution of antigen from the column should be undertaken with the mildest conditions if biological reactivity is to be preserved. High affinity antibodies usually, but not always require the strongest elution conditions (Parham, 1983).

Elution is generally brought about by the induction of a conformational change. Changes in pH (eg. glycine-HCl buffer pH2.2-2.8), polarity lowering agents (eg. dioxane, ethylene glycol), dissociating agents (eg. 5-8M urea, 4-6M guanidine-HCl) and chaotropic agents (eg. thiocyanate) have all been commonly used. Antigens with conformations that depend on environmental conditions, such as temperature or the presence/absence of cofactors, may be bound in one conformation and eluted in another by changing the column environment (eg. temperature). Combinations of reducing and denaturing conditions are likely to dissociate the antibody light and heavy chains, destroying the columns.

The development of an immunoaffinity procedure to concentrate and purify S-PMA from the urine of benzene exposed workers will greatly

facilitate the biomonitoring of benzene exposure. A monoclonal antibody, 3G9, which specifically binds S-PMA, has been generated (chapter 3). This chapter describes the immobilisation of 3G9 to the solid support CNBr activated sepharose, enabling the production of an immunoaffinity column. Immunoreactivity after coupling was assessed by monitoring binding of S-PMA applied to the column in aqueous buffer and urine. The performance of the immunoaffinity column was validated against the solvent extraction GC/MS method described by Boogaard and Van Sittert (1995). The potential of immunoaffinity chromatography/HPLC to determine urinary S-PMA is investigated.

MATERIALS AND METHODS

MATERIALS

REAGENTS

Protein G sepharose Fast Flow and CNBr activated sepharose 4B was supplied by Pharmacia Ltd., Milton Keynes, UK. Sodium acetate (GPR, Hopkin and Williams Ltd., Essex. UK), sodium azide (Sigma Chemical Co., Dorset. UK), d5-S-PMA (Shell Biomedical Laboratory, Rotterdam, Netherlands).

Amicon Centriprep 30 (Amicon Ltd., Stonehouse. UK).

BUFFERS

Coupling buffer consisted of 0.1M sodium carbonate buffer pH 8.3 containing 0.5M NaCl.

Glycine buffer consisted of 0.2M glycine adjusted to pH 8 with 0.2M NaOH, containing 0.5M NaCl

Acetate buffer consisted of 0.1M sodium acetate adjusted to pH 4 with 0.1M acetic acid, containing 0.5M NaCl

1M Tris-HCl consisted of 1M Trizma base adjusted to pH 9 with 1M HCl

PBS sodium azide consisted of PBS containing 0.02% w/v sodium azide

WORKING STANDARDS

ELISA standards (0-2.5ug/ml) in PBS and HPLC standards (0-1ug/ml) in mobile phase (17% acetonitrile, 0.3M phosphate buffer pH2.1) were prepared from a 852ug/ml solution in 1% methanol:PBS.

METHODS

4.8 ANTIBODY PURIFICATION - PROTEIN G AFFINITY CHROMATOGRAPHY

Antibody 3G9 was affinity purified using Protein G sepharose 4 Fast Flow from Pharmacia. A 25ml settled bed volume column was prepared and stored in 20% methanol water at 4°C until use. The column has a binding capacity of approximately 150-300mg of antibody. Before use the column was equilibrated with PBS at 4°C.

Cell culture supernatant (1500ml) was pooled and centrifuged at 4000g for 10 minutes at 4°C to remove cell debris. The supernatant was loaded onto the column and chromatographed in the cold at approximately 2ml/minute. The column was washed with 120ml of PBS and the antibody was eluted with 110ml of 0.1M glycine-HCl pH 2.7. Fractions (10ml) were collected into vials containing 600ul of neutralising buffer (1M Tris-HCl pH 9). The pH of the neutralized fractions was approximately 7.5-8.0. Protein concentration in the collected fractions was determined from optical density measurements at 280nm.

4.9 ANTIBODY CONCENTRATION

Antibody was concentrated (according to the manufacturer's instructions) using an Amicon Centriprep-30 concentrator, a filtration unit containing a membrane (molecular weight cut-off of 30,000), through which buffer is forced by centrifugation.

Briefly, 10-15ml of antibody solution (0.76mg/ml) was centrifuged at

2500rpm for 20 minutes at 25⁰C. The buffer was decanted and the concentration step repeated. Antibody concentration was repeated three times. Concentration of the immunoglobulins was monitored spectrophotometrically at 280nm. Immunoreactivity was assessed by preparing antibody titration curves.

4.10 PREPARATION OF IMMUNOAFFINITY COLUMN

Antibody was covalently attached to a solid support (cyanogen bromide activated sepharose 4B) by a modification of the method described by Pharmacia. Freeze-dried CNBr activated-sepharose 4B (0.21g) was gently mixed with 1mM HCl (15ml) for five minutes at room temperature. The gel which formed was sedimented by centrifugation (2000g for 1 minute), and the supernatant discarded. The rehydration step was repeated three times. The swollen sepharose was washed quickly with coupling buffer (15ml) to remove excess acid.

Immunoglobulin solution (3ml, 1.51 mg/ml) was added to the sepharose and the suspension gently mixed for three hours at room temperature. The sepharose was washed with coupling buffer (15ml), resuspended in glycine buffer (25ml) and gently mixed overnight at 4⁰C to block any remaining active groups on the solid support. The sepharose was washed with coupling buffer (15ml) and acetate buffer (15ml) alternately three times to remove any protein non-covalently bound to the matrix. The gel was washed twice with PBS and a column of approximately 0.5ml settled bed volume was prepared. Finally, the PBS was replaced with PBS containing sodium azide (0.02% w/v) and

the column stored at 4°C until use. Antibody uptake was monitored spectrophotometrically at 280nm.

4.11 IMMUNOAFFINITY CHROMATOGRAPHY

Before use, the immunoaffinity column was allowed to reach room temperature and equilibrated with PBS. Samples were chromatographed at a flow rate of approximately 0.5ml/minute.

S-PMA diluted in PBS (1ug/ml) was loaded (1-20ml) onto the immunoaffinity column. The column was washed with PBS (5ml), and the retentate eluted with methyl alcohol (2ml). Elution fractions, load (1ml), wash (1ml) and retentate (1ml) were collected. The fractions were analysed for the presence of S-PMA by ELISA.

S-PMA in PBS was also diluted to a concentration below the limit of detection of the ELISA (50ug/l) and applied (10ml) to the immunoaffinity column. The column was washed and the retentate eluted as described above. The concentration of S-PMA in the retentate was determined by ELISA.

Mid-stream urine (5, 10 and 20ml) dosed with S-PMA (50, 25 and 5ug/l) were applied to the column for immunoextraction. The retentates were analysed by ELISA and HPLC.

4.12 HPLC ANALYSIS OF S-PMA

HPLC analyses were performed using a C18 reversed phase chromatography column (Hichrome column 10cm x 4.9mm). Mobile phase consisted of 17% acetonitrile, 0.03M phosphate buffer, pH 2.1,

Samples were chromatographed at a flow rate of 1ml/minute. S-PMA was detected spectrophotometrically at 256nm (Gilson model 116). A calibration plot (0.125mg-1.0mg/l) was prepared in mobile phase. Prior to analysis, the immunoaffinity column retentates were concentrated by evaporation and diluted in mobile phase.

4.13 VALIDATION OF THE IMMUNOEXTRACTION PROCEDURE AGAINST A GC/MS METHOD

Chemical workers of the Shell International Petroleum Company are routinely monitored for exposure to benzene. Exposure is determined by the measurement of S-PMA in end of shift urine samples. Urine samples are collected in polythene bottles and acidified to pH 2 with 10% hydrochloric acid (S-PMA under these conditions is the most stable form known to be stable for at least 3 months). The samples are transported to the Biomedical laboratories at Fennis (Rotterdam, Netherlands) where S-PMA is determined by the method of Boogaard.

The performance of the immunoaffinity column was validated against the solvent extraction system described by Boogaard and Van Sittert (1995). Urine containing 12 to 168ug/l (determined after solvent extraction) were determined after immunoextraction using a modification of the procedure described by Boogaard and Van Sittert (1995). GC/MS analysis was performed at the Shell Biomedical Laboratory, Rotterdam.

DETERMINATION OF S-PMA IN THE URINE OF BENZENE EXPOSED WORKERS

To 5ml of urine was added 0.112ug of internal standard d5-S-PMA. Before immunoaffinity chromatography the pH of each urine sample was adjusted to between pH 6.3-6.9. The pH was adjusted by the addition of 2 drops of 6M sodium hydroxide plus 4-12 drops of 1M sodium hydroxide solution.

Before use, the immunoaffinity columns were allowed to reach room temperature and equilibrated with PBS. The urine sample was applied to the immunoaffinity column and chromatographed with a flow rate of approximately 2ml/minute. This flow rate was achieved by placing the columns under a low vacuum. The column was washed with PBS (6ml) and dried under a gentle stream of air. The column retentate was eluted with 95% methanol water (2ml). The concentration of S-PMA and d5 S-PMA in the sample was determined by GC/MS.

DERIVATIZATION OF S-PMA FOR GC/MS DETERMINATION

For GC the mercapturic acids were derivatised as follows. The 95% methanol/water was evaporated under a gentle stream of nitrogen at 45°C, and the residue dried by warming above an infra red lamp for 1 hour. The dry sample was methylated by the addition of 2ml of methanol/1.25M HCl. Methylating agent was prepared by bubbling 46g of HCl gas through 1 litre of methanol, and the pH checked by titration with sodium hydroxide. Following methylation at room temperature for 30 minutes the sample was evaporated to dryness under a gentle stream of nitrogen at 45°C. The residue was dissolved

in 100ul of dichloromethane and analysed by GC/MS with selective ion recording.

GC/MS DETERMINATION OF S-PMA-METHYESTER

It was not possible to analyse the samples using the conditions described by Boogaard. The modified GC/MS conditions were as follows: the column was a fused silica capillary column (60m x 0.22mm) coated with DB-1 (Durabond, film thickness 0.1 micrometres). Helium was used as the carrier gas, 170kPa (flow rate of approximately 1ml/minute). The injector temperature was initially 150⁰C. After the sample was loaded this temperature was held for 1 second then increased to 250⁰C by 12⁰C/second. The injection volume was 1ul splitless. The oven programme was initially 50⁰C for 1 minute followed by a 10⁰C/minute increase to 300⁰C. This temperature was held for 7.5 minutes.

Electron impact ionisation was performed with the following conditions: ion source temperature 180⁰C, ionisation potential 70eV and multiplier voltage autotune plus 800 eV. For quantification of S-PMA and d5-S-PMA the ions at m/z 194 and 199 (fragment of S-PMA/d5-S-PMA methyl ester) were monitored. Qualification ions at m/z 253 and 254 respectively were also recorded.

A calibration curve (fig. 4.3) was prepared in blank urine (0-100ug/l). The ratio of the methyl esters of S-PMA and d5-S-PMA internal standard was plotted against the response ratio of m/z 194 and m/z 199.

The concentration of the S-PMA in a urine sample (C_u) was calculated from the equation:

$$C_u = \frac{A_u}{A_B} \times \frac{C_B}{F}$$

F, a factor of relative sensitivity (m/z 194 : m/z 199) is approximately 1 and can be calculated from the linear equation:

$$\frac{A_P}{(A_B - A_{BO})} \times C_B = C_P \times F + \frac{A_{PO}}{(A_B - A_{BO})} \times C_B$$

when

A_B = area/peak height of m/z 199 in calibration or unknown urine

A_{BO} = area/peak height of m/z 199 in blank calibration urine without internal standard

A_P = area/peak height of m/z 194 in calibration urine

A_{PO} = area/peak height of m/z 194 in blank calibration urine with internal standard

A_u = area/peak height of m/z in the unknown urine

C_B = concentration of internal standard d5-S-PMA in calibration or unknown urine.

C_P = concentration of S-PMA in the calibration urine

C_u = concentration of S-PMA in the unknown urine

4.14 IMMUNOAFFINITY CHROMATOGRAPHY/HPLC ANALYSES OF URINARY S-PMA

Finally, the concentration of S-PMA in urine samples collected from benzene exposed workers was determined by immunoaffinity

chromatography/HPLC. The concentration of S-PMA in the urine samples (30-1152ug/l) had been determined previously by GC/MS (method of Boogaard and Van Sittert 1995).

Urine samples (1-5ml) were applied to the immunoaffinity column and chromatographed as described above. Prior to analyses by HPLC, the immunoaffinity chromatography retentates were concentrated by evaporation, under a stream of nitrogen gas, and diluted in mobile phase (500ul).

RESULTS

4.15 ANTIBODY PURIFICATION AND CONCENTRATION

Protein G purification yielded 30ug of 3G9 per ml of supernatant. From the immunoreactivity studies, this corresponded to a 16 fold increase in concentration (table 4.1). A further 25 fold increase in 3G9 concentration was achieved with the Amicon Centriprep 30. It was calculated from OD measurements that 96% of the antibody was recovered after membrane filtration.

Table 4.1 Antibody 3G9 Purification v Immunoreactivity

FRACTION	TITRE
CULTURE SUPERNATANT	1:2500
AFTER PROTEIN G PURIFICATION	1:40000
AFTER CONCENTRATION	1:1000000

4.16 PREPARATION OF IMMUNOAFFINITY COLUMNS

4.06mg (89%) of 3G9 was coupled to 0.21g (0.75ml of swollen gel) of CNBr activated sepharose. No protein was detected in the final coupling/acetate washes (O.D. readings at 280nm) indicating that all the antibody was covalently attached to the solid support.

4.17 IMMUNOAFFINITY CHROMATOGRAPHY

The immunoaffinity column had a maximum binding capacity of 1.5ug of

S-PMA. No S-PMA was observed in the final washes when the fractions were analysed by ELISA. 0.54ug (108%) of the S-PMA was recovered after immunoenrichment of a solution (10ml) containing 50ug/l of analyte. Recoveries of 80% (SD=6.7, n=6) were obtained after immunoaffinity chromatography of a 5ml urine sample containing 50ug/l of S-PMA. Recoveries of 94% and 80% were obtained after immunoaffinity chromatography of 10 and 20ml of urine containing 25 and 5ug/l of S-PMA respectively.

4.18 DETERMINATION OF S-PMA BY GC/MS

The S-PMA and the internal standard (d5-S-PMA) "selected ions" (m/z 194 and 199) and "qualifier" ions (m/z 253 and 258) had a ratio of 10 to 1. A typical calibration, the ratio of methyl ester of S-PMA and d5-S-PMA internal standard plotted against the response ratio m/z 194 and 199, is shown in figure 4.3.

Twenty three urine samples containing 12-168ug/l S-PMA analysed by the method of Boogaard were analysed by immunoaffinity chromatography - GC/MS. The correlation coefficient between the two methods was 0.98, figure 4.4. Recovery of S-PMA after acidification and neutralization of the urine was 28.3% (SD=6.2, n=23). GC/MS chromatograms of immunoextracted samples contained fewer peaks than those analysed by the method of Boogaard.

Typical examples of the fragmentographs obtained from immunoaffinity prepared samples are shown in figures 4.5 to 4.8. Fragmentographs of the same samples prepared by the method of Boogaard are shown in figures 4.9 to 4.12. Figures 4.5 and 4.9 are the fragmentograph of a

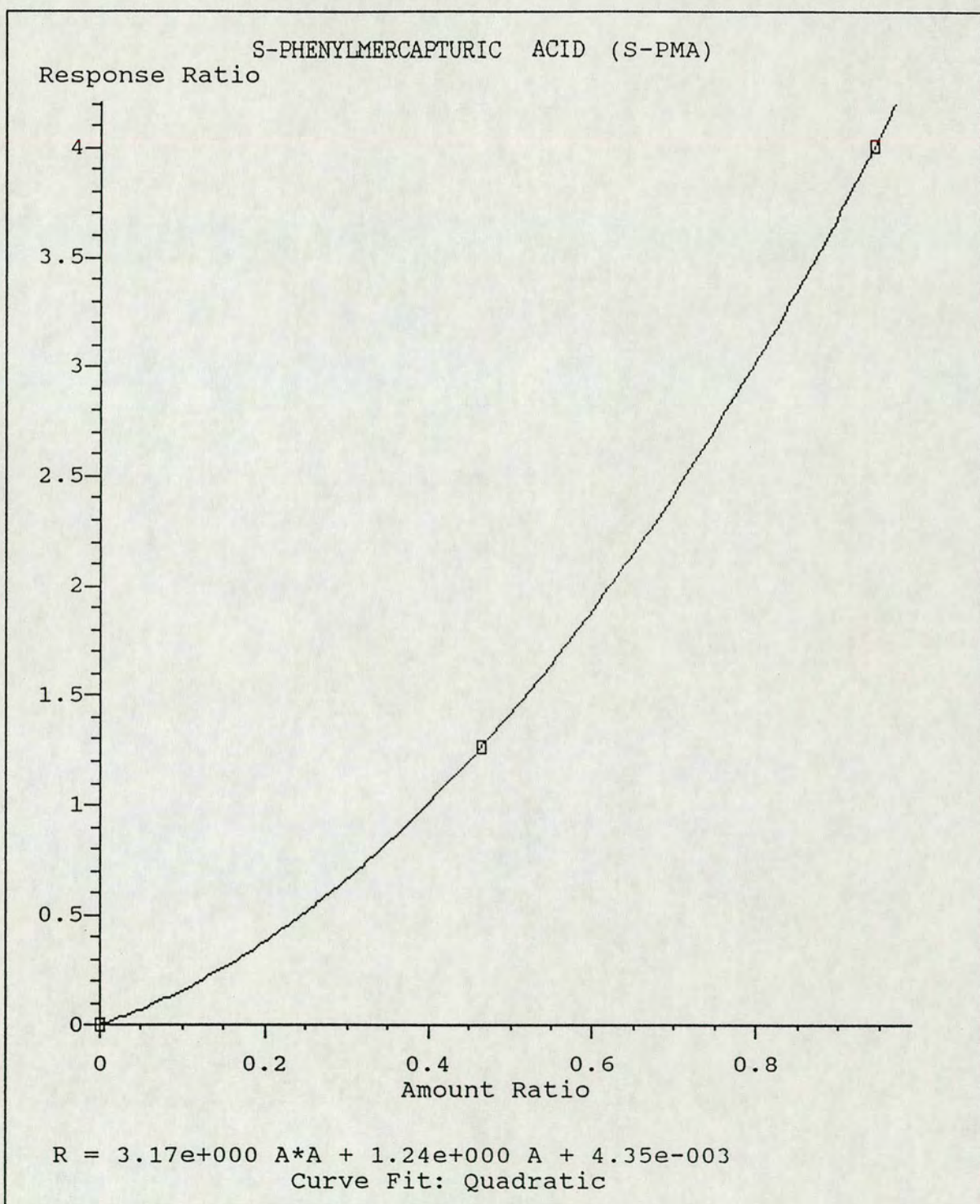
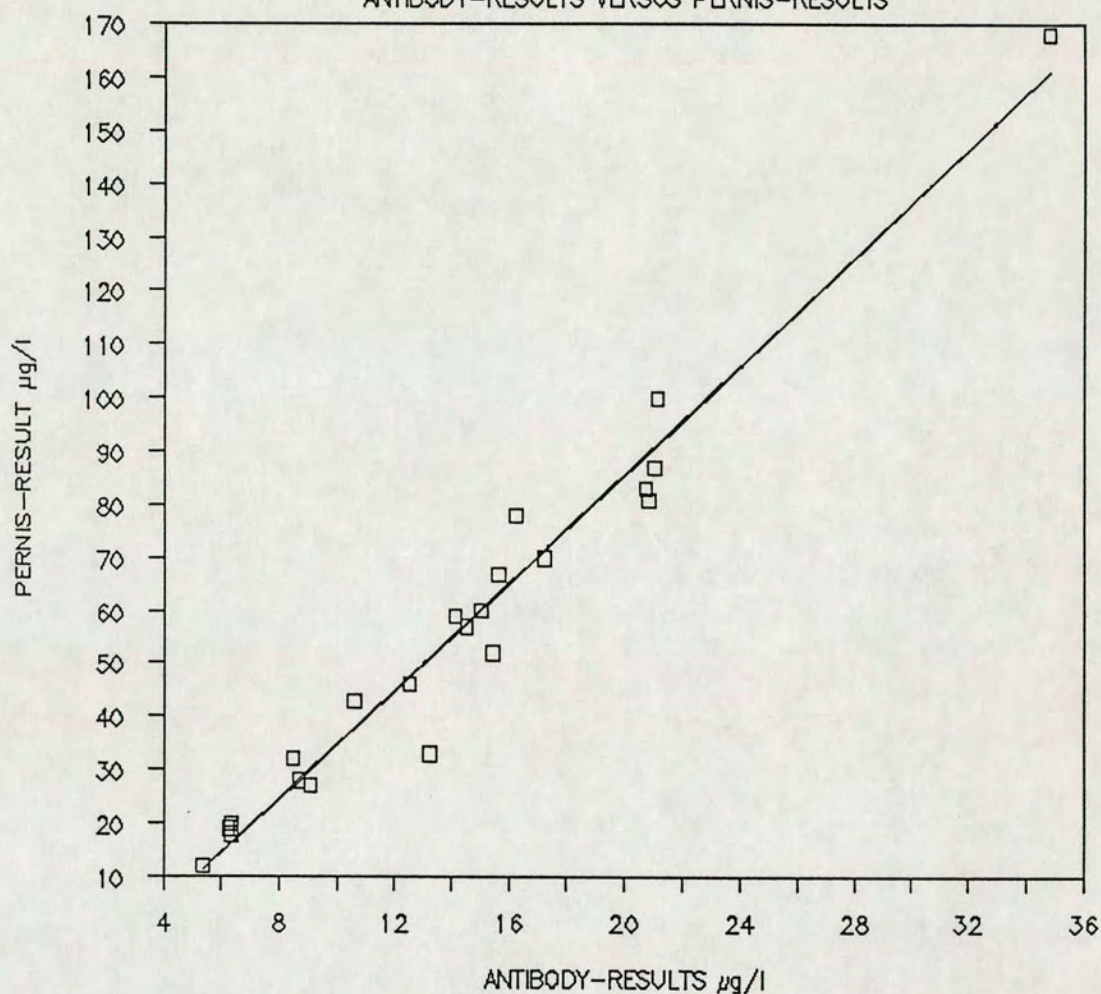


Figure 4.3 GC/MS S-PMA calibration plot.

CORRELATION-GRAPHICS

ANTIBODY-RESULTS VERSUS PERNIS-RESULTS



Regression Output:

Constant	-15.9779	
Std Err of Y Est	6.704347	
R Squared	0.966306	0.983008
No. of Observations	23	
Degrees of Freedom	21	
X Coefficient(s)	5.084379	
Std Err of Coef.	0.207179	

Figure 4.4 Correlation between antibody method and GC/MS method.

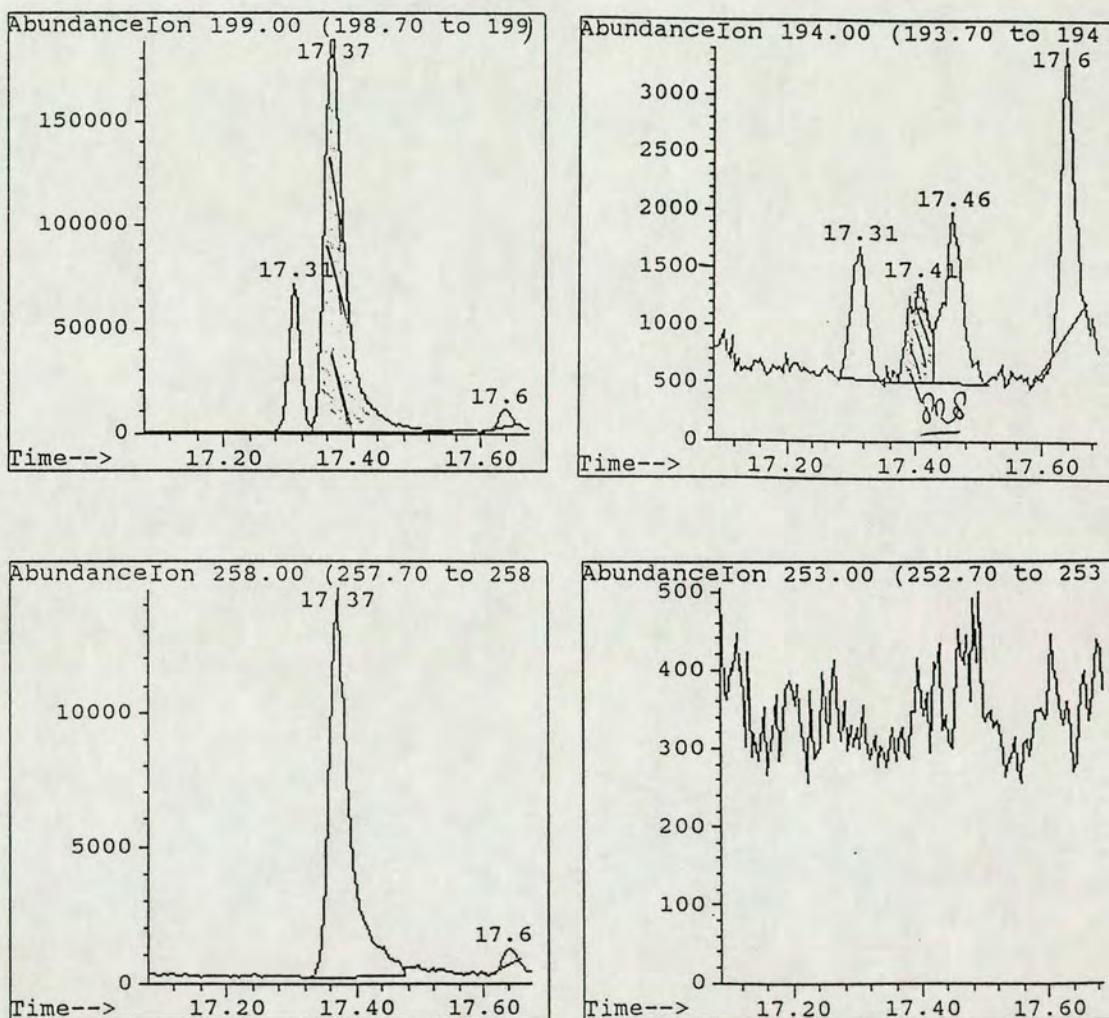


Figure 4.5

Fragmentograph of a urine containing 0.7 μ g/l of S-PMA. Sample was prepared by immunoaffinity chromatography. The upper right panel shows the fragmentograph of the main ion for S-PMA-methyester (m/z 194) and the lower right panel the ion m/z 253 which serves as a "qualifier ion". The upper left panel shows the main ion (m/z 199) and the lower left panel the "qualifier ion" (m/z 258) for the internal standard d5-S-PMA-methyester.

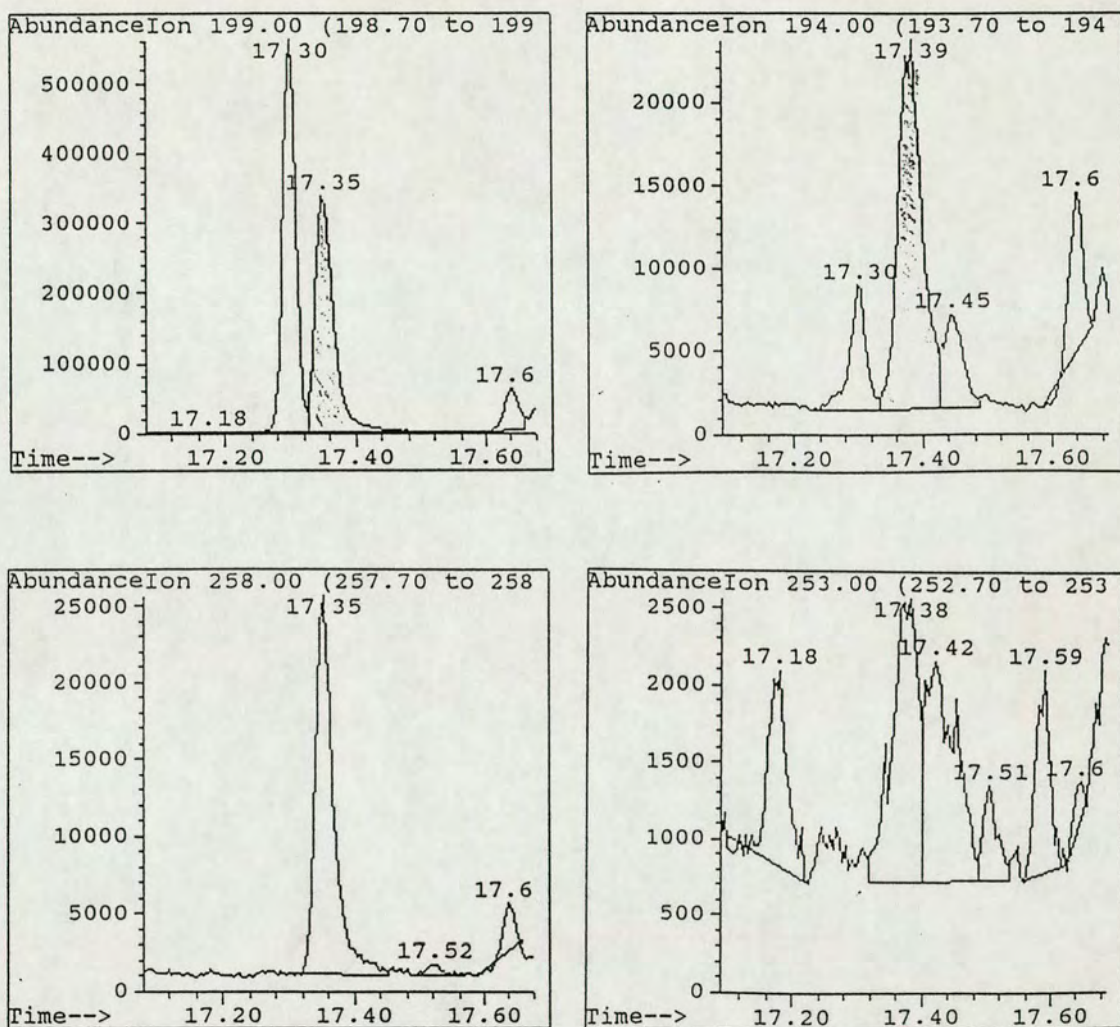


Figure 4.6

Fragmentograph of a urine containing 7ug/l of S-PMA. Sample was prepared by immunoaffinity chromatography. The upper right panel shows the fragmentograph of the main ion for S-PMA-methyester (m/z 194) and the lower right panel the ion m/z 253 which serves as a "qualifier ion". The upper left panel shows the main ion (m/z 199) and the lower left panel the "qualifier ion" (m/z 258) for the internal standard d_5 -S-PMA-methyester.

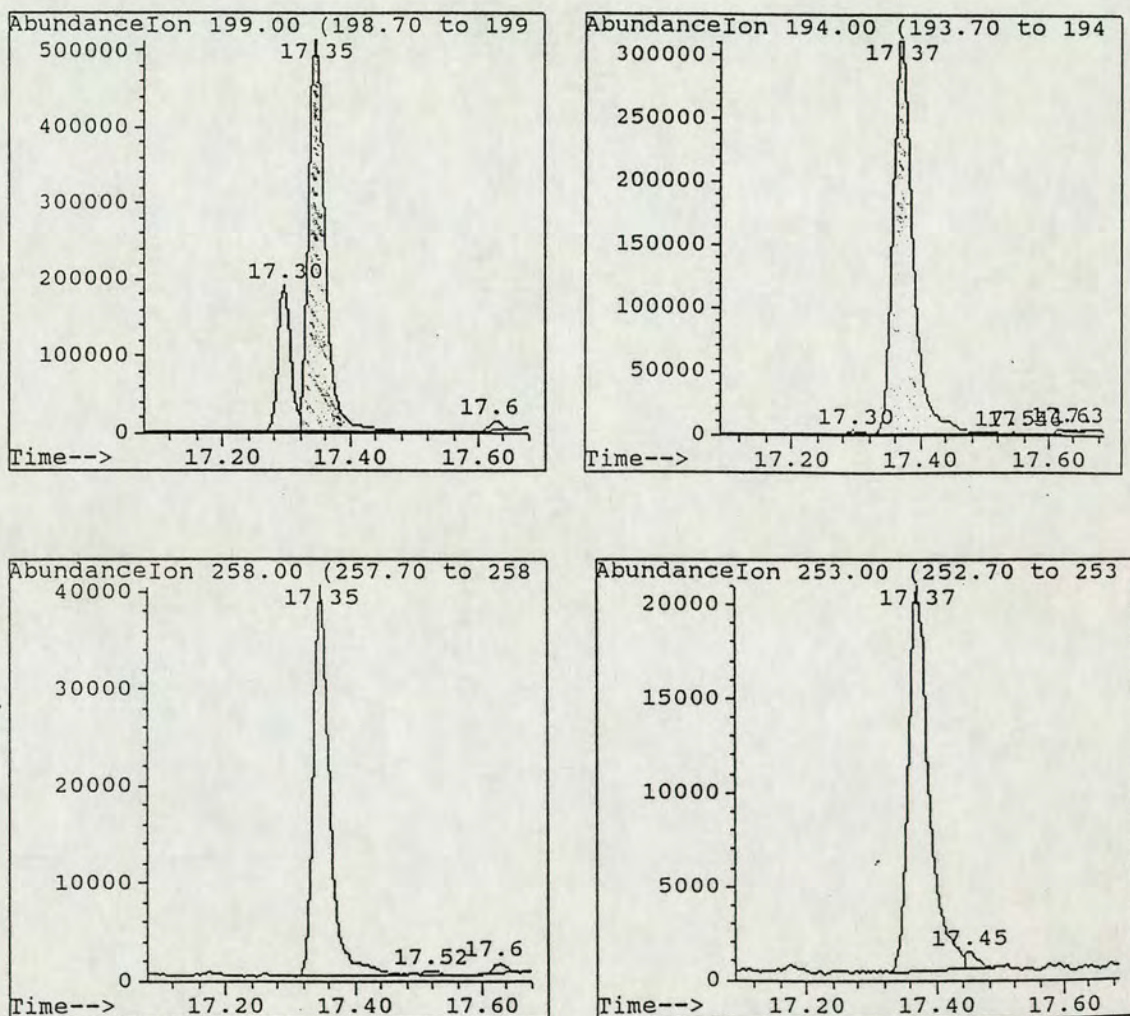


Figure 4.7.

Fragmentograph of a urine containing 18ug/l of S-PMA. Sample was prepared by immunoaffinity chromatography. The upper right panel shows the fragmentograph of the main ion for S-PMA-methyester (m/z 194) and the lower right panel the ion m/z 253 which serves as a "qualifier ion". The upper left panel shows the main ion (m/z 199) and the lower left panel the "qualifier ion" (m/z 258) for the internal standard d5-S-PMA-methyester.

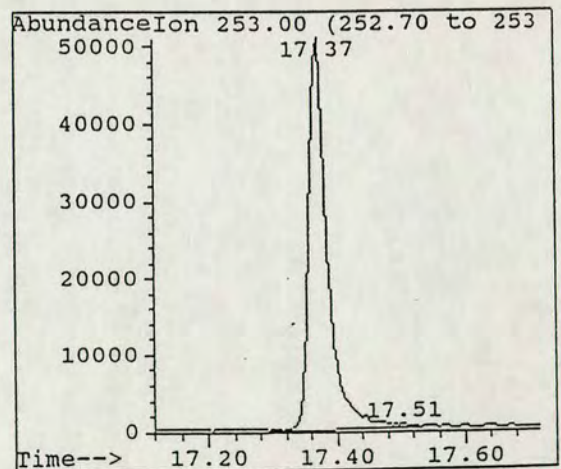
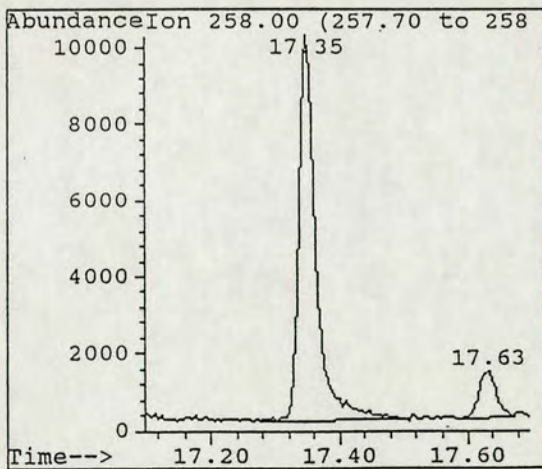
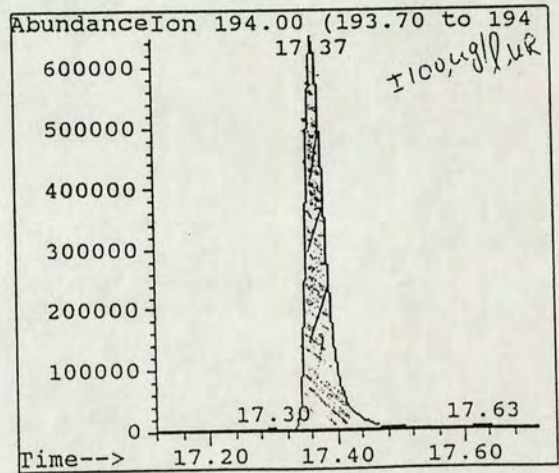
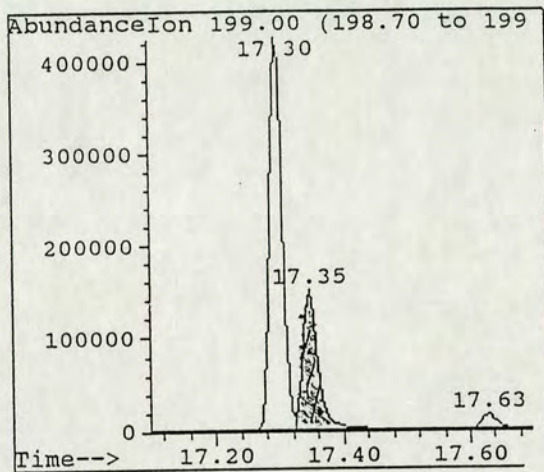


Figure 4.8.

Fragmentograph of a urine containing 100ug/l of S-PMA. Sample was prepared by immunoaffinity chromatography. The upper right panel shows the fragmentograph of the main ion for S-PMA-methyester (m/z 194) and the lower right panel the ion m/z 253 which serves as a "qualifier ion". The upper left panel shows the main ion (m/z 199) and the lower left panel the "qualifier ion" (m/z 258) for the internal standard d_5 -S-PMA-methyester.

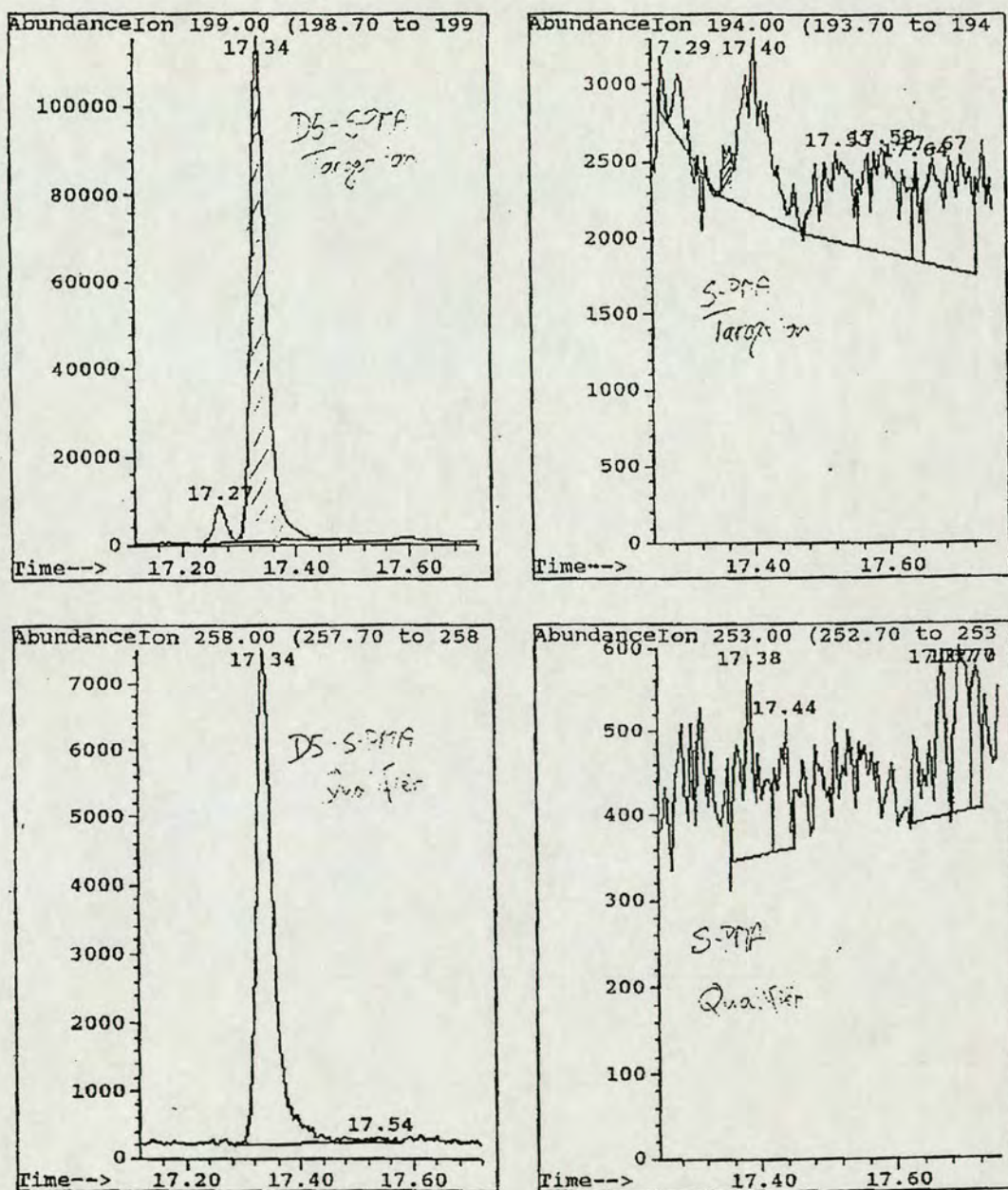


Figure 4.9.

Fragmentograph of a urine containing 0.7ug/l of S-PMA. Sample was prepared by the method of Boogaard. The upper right panel shows the fragmentograph of the main ion for S-PMA-methyester(m/z 194) and the lower right panel the ion m/z 253 which serves as a "qualifier ion". The upper left panel shows the main ion (m/z 199) and the lower left panel the "qualifier ion" (m/z 258) for the internal standard d5-S-PMA-methyester.

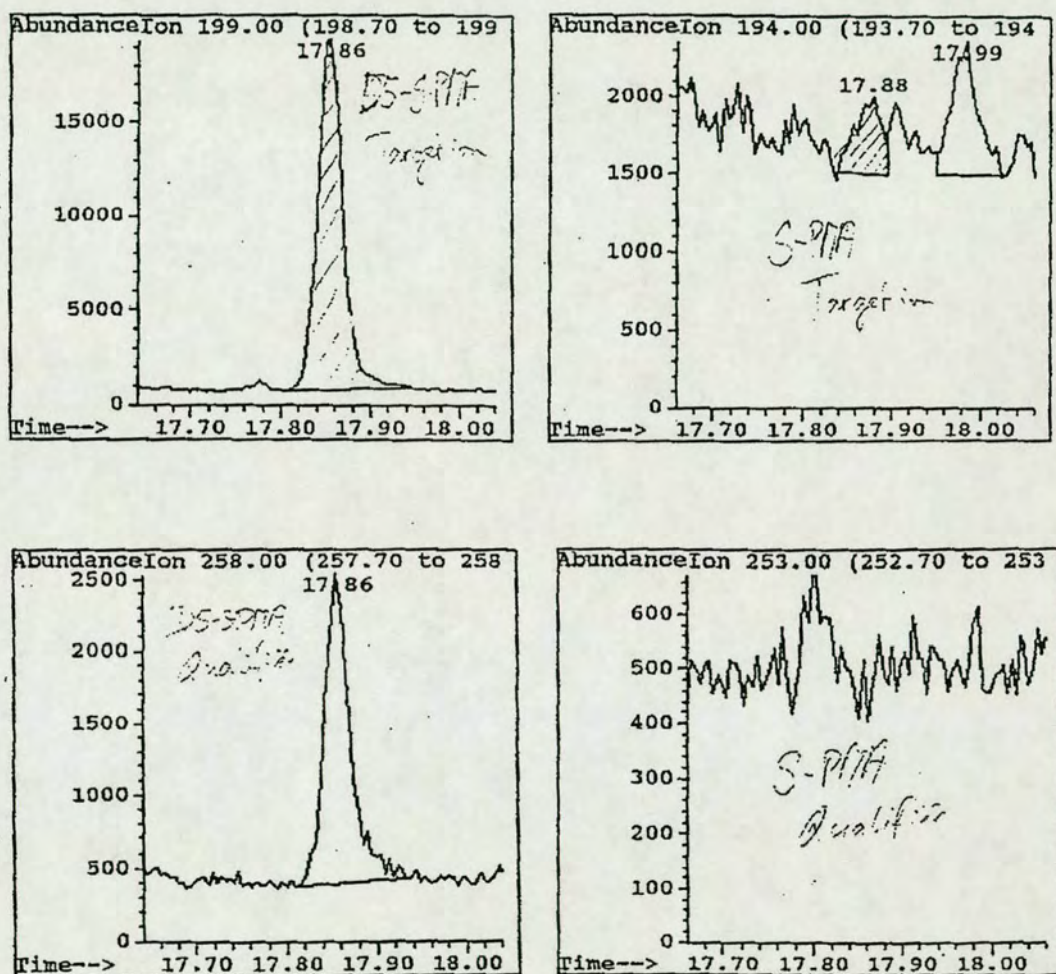


Figure 4.10.

Fragmentograph of a urine containing 7ug/l of S-PMA. Sample was prepared by the method of Boogaard. The upper right panel shows the fragmentograph of the main ion for S-PMA-methyester (m/z 194) and the lower right panel the ion m/z 253 which serves as a "qualifier ion". The upper left panel shows the main ion (m/z 199) and the lower left panel the "qualifier ion" (m/z 258) for the internal standard d5-S-PMA-methyester.

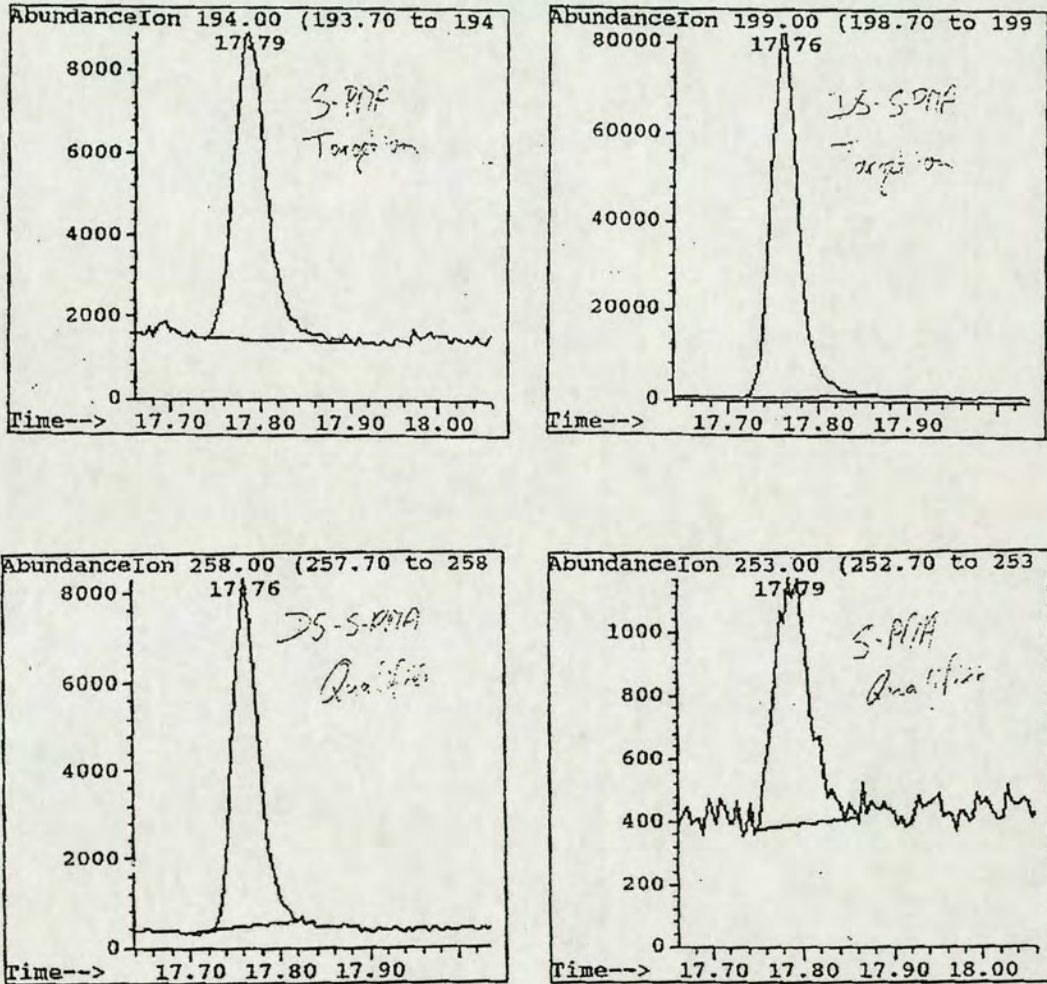


Figure 4.11.

Fragmentograph of a urine containing 18ug/l of S-PMA. Sample was prepared by the method of Boogaard. The upper right panel shows the fragmentograph of the main ion for S-PMA-methyester (m/z 194) and the lower right panel the ion m/z 253 which serves as a "qualifier ion". The upper left panel shows the main ion (m/z 199) and the lower left panel the "qualifier ion" (m/z 258) for the internal standard d5-S-PMA-methyester.

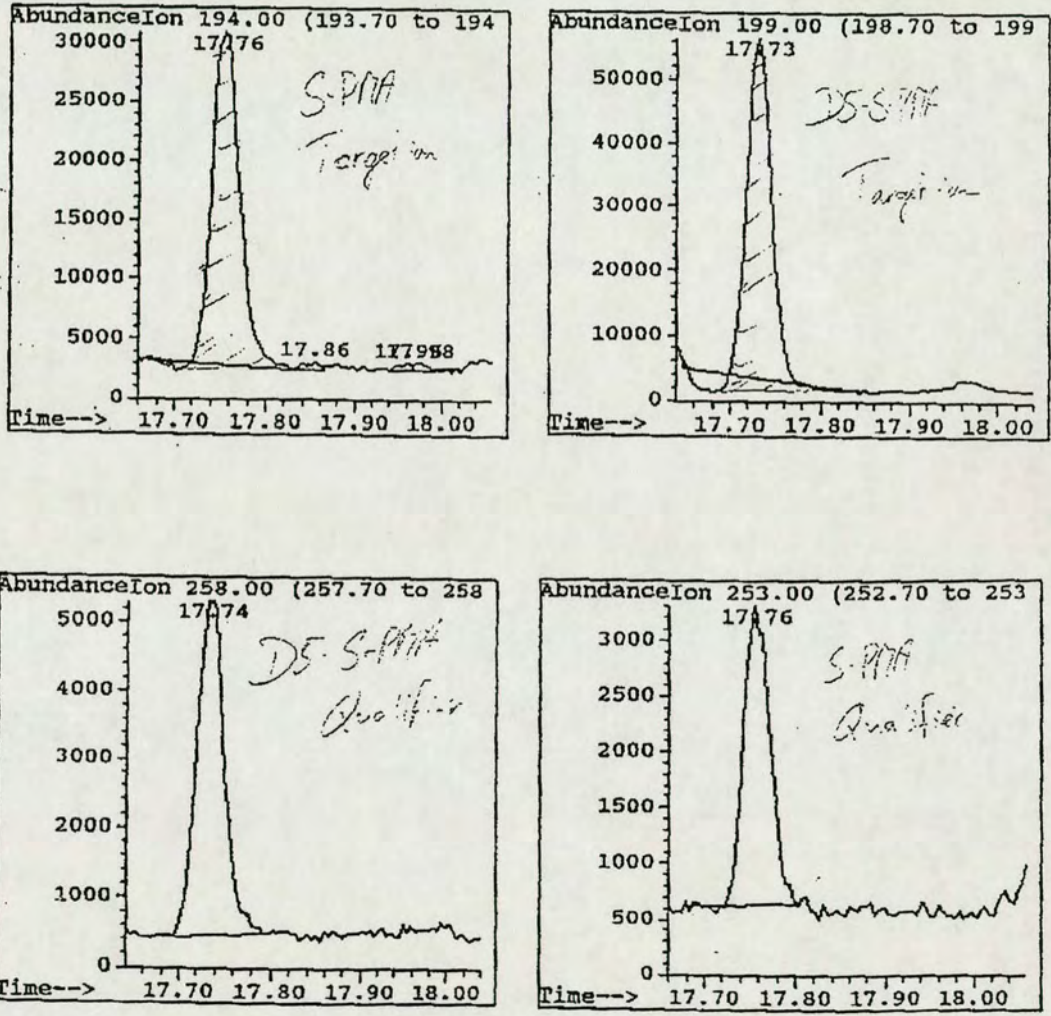


Figure 4.12.

Fragmentograph of a urine containing 100ug/l of S-PMA. Sample was prepared by the method of Boogaard. The upper right panel shows the fragmentograph of the main ion for S-PMA-methyester (m/z 194) and the lower right panel the ion m/z 253 which serves as a "qualifier ion". The upper left panel shows the main ion (m/z 199) and the lower left panel the "qualifier ion" (m/z 258) for the internal standard d5-S-PMA-methyester.

non-exposed person, the background level of S-PMA is 0.7ug/l. Figures 4.6-4.8 and 4.10-4.12 are from urines containing 7, 18 and 100ug/l of S-PMA. The retention times of the S-PMA and d5-S-PMA methyl ester ions, m/z 194 and 199, are between 17.37-17.41 and 17.35-17.37 minutes respectively. The "qualifier ions", m/z 253 and 258, are also shown.

HPLC DETERMINATION OF URINARY S-PMA

A linear calibration plot (0.125-1.0mg/l) was obtained with standards prepared in mobile phase. The HPLC limit of detection was 50ug/l. The results of the HPLC analyses of immunoaffinity column eluates from the urine of workers exposed to benzene are shown in Table 4.2. The corresponding data obtained by GC/MS are also presented. The concentration of urinary S-PMA ranged from 30 to 1152ug/l. The correlation coefficient was 0.964 (n=6). A typical HPLC chromatograph is illustrated in figure 4.13. S-PMA has a retention time of 14 minutes under the HPLC conditions employed. A mixed injection of sample and authentic S-PMA co-eluted.

Table 4.2 Comparison of S-PMA Concentrations Determined by GC/MS and Immunoaffinity Chromatography (IAC)/HPLC in the Urine of Benzene Exposed Workers

GC/MS (ug/l)	IAC/HPLC (ug/l)
1152	1157
580	297
492	476
154	143
60	63
30	32

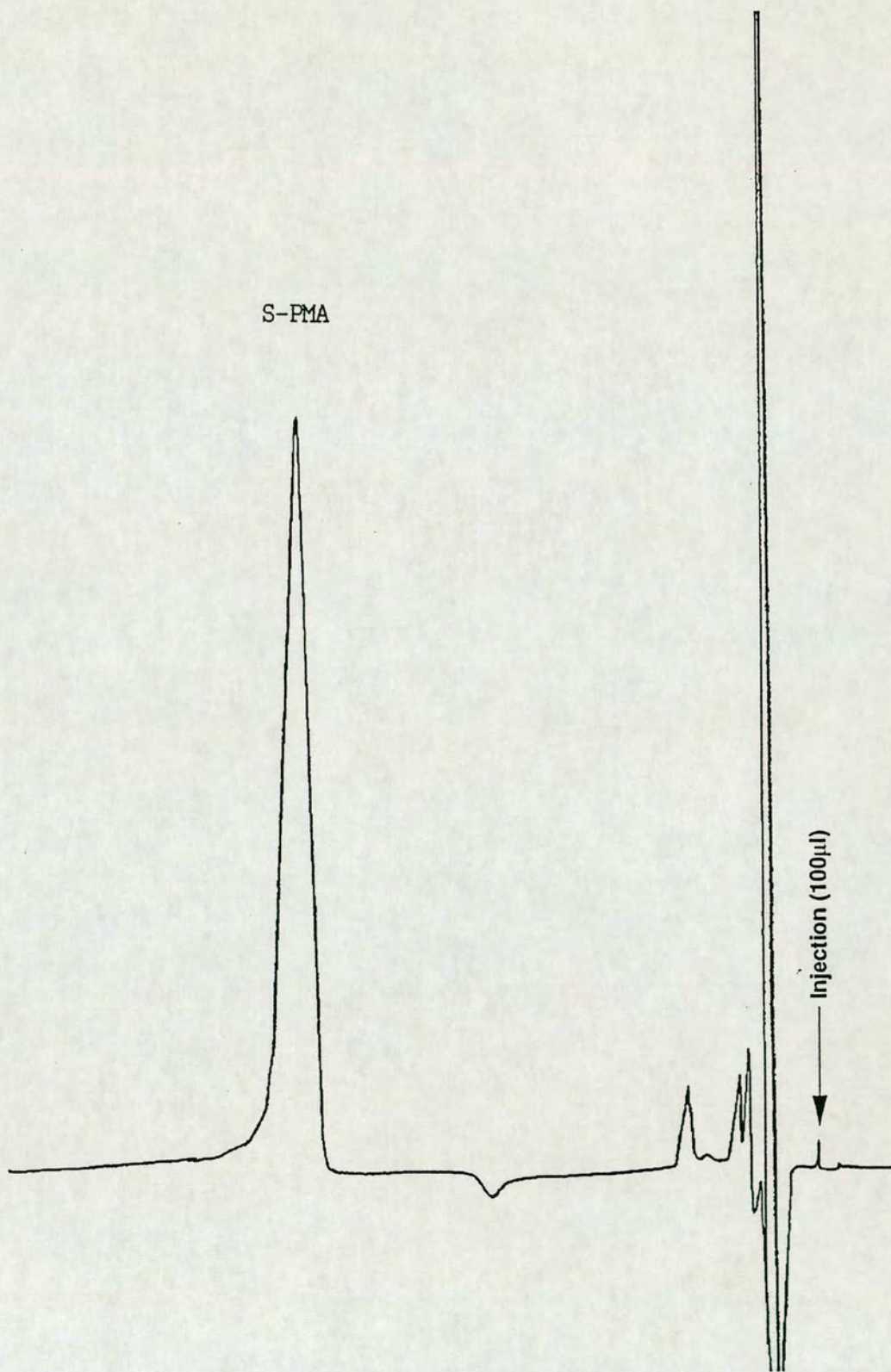


Figure 4.13 HPLC chromatogram of S-PMA after immunoextraction from the urine of a benzene exposed worker. The urine contained 1152ug/l of S-PMA determined by GC/MS.

DISCUSSION

A monoclonal antibody, 3G9, which specifically binds S-PMA, has been generated. 3G9 retains its immunoreactivity after coupling to a solid support, enabling the production of an immunoaffinity column. The chromatography column can be used to selectively extract low levels of S-PMA (0.7ug/l) from urine. The performance of the immunoaffinity column at low urinary concentrations of S-PMA (12-168ug/l) has been validated in a comparative study with the method of Boogaard and Van Sittert (1995). The two methods show excellent agreement. The correlation coefficient was 0.98 (n=23) over this range of S-PMA. Furthermore, bioconcentration of S-PMA from the urine of benzene exposed workers has permitted the quantification of S-PMA by HPLC at 8 hour TWA airbourne exposures of around 1ppm.

Immunoreactivity was assessed by monitoring binding of S-PMA applied to the column in aqueous buffer. The column adsorbed 1.5ug of S-PMA and the analyte did not appear to leach from the column during washing. High recoveries (up to 100% of the load) were obtained where the retentate was eluted from the column with methanol. Moreover, the column retained functionality even after a very high percentage (95%) solvent elution step. With repeated use (10 times) there was no noticeable loss in column performance. Observations show no evidence of changes in the antibody binding characteristics with time.

The column also proved to be effective in selectively adsorbing and concentrating S-PMA from mid-stream urine. Loading analyte in this matrix appeared to have no detrimental effect on column performance.

Furthermore, recovery of analyte appears to be independent of sample volume. At present urine samples are collected in polythene bottles and acidified to pH 2, at this pH S-PMA has been shown to be stable for at least one month (Van Sittert et al, 1993). Acidification and neutralisation of urine exercised a strong effect on column performance. Recovery of S-PMA (28.3%) was lower than expected and is probably due to high levels of salt disrupting antigen antibody binding. Improving sample recovery will further improve assay sensitivity. Recovery may be improved if urines are desalted prior to extraction. Alternatively, samples may be stored frozen rather than at acidic pH after collection.

The amount of antibody coupled to the solid support (5.4ug/ml gel) was less than the quoted binding capacity (8-10mg/ml gel). However antibody uptake (89%) is in the optimum range (80-90%) described by Pfeiffer et al (1992). In this study, no further attempts have been made to optimise the antibody coupling chemistry or the chromatography conditions and additional improvements in assay performance may be possible.

We have shown that 3G9 is predominantly reactive with S-PMA. GC/MS chromatograms of immunoextracted samples contained fewer peaks than those samples analysed by the method of Boogaard. Due to the loss of sample matrix it was necessary to modify the reported GC conditions. Changes were made to the injection protocol in order to prevent loss of sample during the solvent purge.

After immunoextraction background levels of S-PMA could be measured (fig. 4.5). In non-exposed populations the concentration of S-PMA

is in the range 0.7-2.3ug/l. To compensate for differences in individual kidney filtration rates the amount of S-PMA is normally expressed per gram of creatinine. Figures 4.6, 4.7 and 4.8 are of urines containing 7, 18 and 100ug/l S-PMA respectively. Creatinine concentrations have previously been determined. The concentrations of S-PMA per gram of creatinine are 4, 31 and 44ug/g respectively. The mean urinary levels of S-PMA in smokers and non-smokers are 3.61 and 1.99ug/g of creatinine. End of shift urine samples from workers exposed to 1ppm of benzene, 8 hour TWA, have an average S-PMA concentration of 47ug/g creatinine.

The limit of detection of the present assay for S-PMA is 0.3ppm, 8 hour TWA, of benzene (Boogaard and Van Sittert 1995). The sensitivity of the GC/MS assay is limited by the purity of the sample. Immunopurification reduces the background noise in the MS fragmentographs (figures 4.6-4.8 and 4.10-4.12) and, therefore, the data demonstrates the potential of immunoaffinity chromatography to increase GC/MS assay sensitivity. It is estimated that immunoaffinity chromatography gives a ten fold increase in GC/MS assay sensitivity.

Bioconcentration has facilitated the measurement of S-PMA by HPLC from the urine of benzene exposed workers. Results obtained by immunoaffinity chromatography/HPLC correlate (correlation coefficient 0.964) with those obtained by GC/MS (Table 4.2) over a range of values (30-1152ug/l). GC/MS has shown that 3G9 is predominantly reactive with S-PMA. The excellent agreement between the data derived by post-immunoenrichment HPLC and those obtained by

GC/MS analyses also suggest that no endogenous components of urine are recognised by 3G9 or interfere with antibody-analyte binding. The HPLC chromatogram (figure 4.13) is free from interfering peaks. Sensitivity could be further improved by increasing the volume of urine subjected to immunopurification.

Immunoaffinity chromatography/HPLC analyses are simple and easy to perform compared with GC/MS. It is estimated that 75 samples per a day can be analysed by IAC/HPLC. In addition, HPLC determination can be readily performed in many laboratories, unlike GC/MS analyses which restrict the method to use in sophisticated laboratories. Our data demonstrates the potential utility of immunoaffinity chromatography/HPLC in monitoring exposure to benzene.

Further improvements in the immunoaffinity/HPLC analysis of urinary S-PMA were made by changing the HPLC mobile phase to water:methanol:acetic acid, 70:30:1. This eliminates sample preparation after immunoextraction and allows the direct determination of S-PMA in column eluants, thereby reducing both the number of stages where sample loss may occur and assay time. In addition, to improve assay reliability an internal standard - S-phenyl-N-trifluoroacetyl-L-cysteine - has been synthesised. Preliminary studies indicate S-phenyl-N-trifluoroacetyl-L-cysteine is bound by antibody 3G9 and can be extracted from a spiked urine by immunoaffinity chromatography. The retention time of S-PMA and S-phenyl-N-trifluoroacetyl-L-cysteine was 11 and 22 minutes respectively when water:methanol:acetic acid was used as the mobile phase.

Having developed and validated a simple laboratory assay for the measurement of urinary S-PMA, future investigations will focus on the production of a field kit which will enable simple and rapid tests for exposure monitoring by operators in the field.

CHAPTER FIVE

THE DETERMINATION OF HAEMOGLOBIN ADDUCTS

INTRODUCTION

Compounds which directly or indirectly give rise to mercapturic acids are also able to react with the nucleophilic centres in protein molecules. The determination of protein adducts is therefore an alternative approach to determining mercapturic acids. Due to the difficulties encountered in raising useful monoclonal antibodies to 2-HEMA (chapters 2 and 3) the determination of 2-(hydroxyethyl) adducted proteins was investigated

5.1 PROTEIN ADDUCTS AND RISK ASSESSMENT

Proteins are macromolecules which contain nucleophilic centres (eg. the sulphhydryl group of cysteine, methione sulfurs, the N1 and N3 atoms of histidine, the amino group in lysine, the aspartic and glutamic carboxylates and the N terminal amino group) which are able to react with electrophiles. In 1974, Ehrenberg et al first suggested the use of proteins as nucleophiles for use in dose monitoring studies and demonstrated that alkylation of tissue proteins could be used to measure the genetic risk from electrophiles in the environment. Mice were exposed to air containing ethylene oxide and the hydroxyethylation of protein and DNA in several tissues was measured. The level of binding was very similar in the tissues examined and the ratio of protein and DNA binding was consistently 2.2-2.8 : 1.

Furthermore, Ehrenberg and his colleagues translated tissue dose into risk equivalents by expressing the genetic risks of

environmental chemicals in the framework of low LET (Low energy transmission) radiation. Quantifying risks from various sources in the same unit - the "rad-equivalent"- Ehrenberg facilitated the comparison of risks, of alternatives in risk-benefit evaluations and the summation of risks of various origins. On the basis of dose-effect curves of ethylene oxide and X-rays, in barley, a tissue dose of 1mM.h of ethylene oxide equals 80 rads of low LET radiation. For a review of the 'rad-equivalence' concept see Tornqvist et al (1991).

5.2 HAEMOGLOBIN ADDUCTS

For applications in human tissue dosimetry the chosen adduct must be stable and determinable by non-nuclear methods. The protein should also be abundant and readily available for sampling. Haemoglobin fulfils these criteria and has been shown to be suitable for the evaluation of genetic risks of alkylating agents (Osterman-Golker et al 1976).

Exposing mice to either ethylene oxide or dimethylnitrosamine, a carcinogen which needs metabolic activation, Osterman-Golker demonstrated that haemoglobin alkylation could be used to measure exposure to both primary alkylating agents and compounds that are metabolised to alkylating agents. They also demonstrated that the life time of haemoglobin (about 4 months in man) was not affected by alkylation making it suitable for monitoring both acute and chronic exposure. Several direct and indirect alkylating agents have been shown to give rise to hydroxyethylated haemoglobin: ethene (Ehrenberg et al 1977), vinyl chloride (Osterman-Golker et al 1977)

and ethyl methanesulfonate (Truong et al 1978). The analysis of haemoglobin as a dose monitor for alkylating and arylating agents has been reviewed by Neumann (1984).

5.3 DETERMINATION OF HAEMOGLOBIN ADDUCTS

In their initial studies, Ehrenberg et al (1974) determined the number of adducted histidine molecules in haemoglobin by GC/MS (after protein hydrolysis and adduct enrichment). The method is very sensitive and background levels of adducted histidine were reported in populations not exposed to alkylating agents (Van Sittert 1985). However, these methods were laborious, expensive and not suitable for monitoring large populations. More suitable methods for biomonitoring are based on the determination of adducts formed by reaction with the amino groups of the N-terminal valine residues of the α and β chains of haemoglobin. (For a review see Britton et al, 1991).

5.4 THE EDMAN DEGRADATION REACTION

The N-terminal adducted valines of the α and β chains of haemoglobin are the major haemoglobin adducts formed on exposure to alkylating agents. Adducted N-terminal valines can be quantified by the Edman degradation reaction. Edman degradation sequentially removes one residue at a time from the amino terminus of a peptide. In an Edman degradation reaction, phenyl isocyanate is reacted with uncharged terminal amino groups to form a phenylthiocarbamoyl derivative which is liberated under mildly acidic conditions as the

cyclic phenylthiohydantoin amino acid (Figure 5.1). The phenylthiohydantoin derivative can be quantified after separation by GC. In 1984 Jensen et al showed that the alkylated N-terminal valine was selectively split off from haemoglobin under neutral-alkaline conditions leaving unadducted valine attached to the protein. The method was rapid but detection of the phenyl isocyanate conjugate lacked the desired sensitivity for biomedical monitoring (due to the low response of the flame ionisation or electrochemical detectors).

5.5 THE MODIFIED EDMAN DEGRADATION REACTION

A breakthrough came two years later when M. Tornqvist et al (1986) substituted the conventional sequencing reagent, phenyl isothiocyanate, with pentafluorophenyl isothiocyanate (PFPTC, Figure 5.2). Pentafluorophenylthiohydantoin (PFPTH) derivatives (Figure 5.2) could be detected by GC/MS (negative ion chemical ionisation) with a sensitivity of 1-10pmol hydroxyethylvaline/g Hb. This detection limit corresponds to a degree of alkylation obtained from exposure to ethylene oxide at an average level of 0.01 ppm 40hour/week for four months, the life span of haemoglobin in man (Tornqvist 1985).

With the modified Edman degradation procedure it has also been possible to monitor epoxide doses from exposure to ethene. For example, ethene is used to ripen bananas and workers in this area had adduct levels elevated by 25 pmol/g globin. Similarly, ethene in cigarette smoke raised the number of hydroxyethylvaline adducts, on average, by 85 pmol/g globin for people smoking 10 cigarettes per day (Tornqvist 1991).

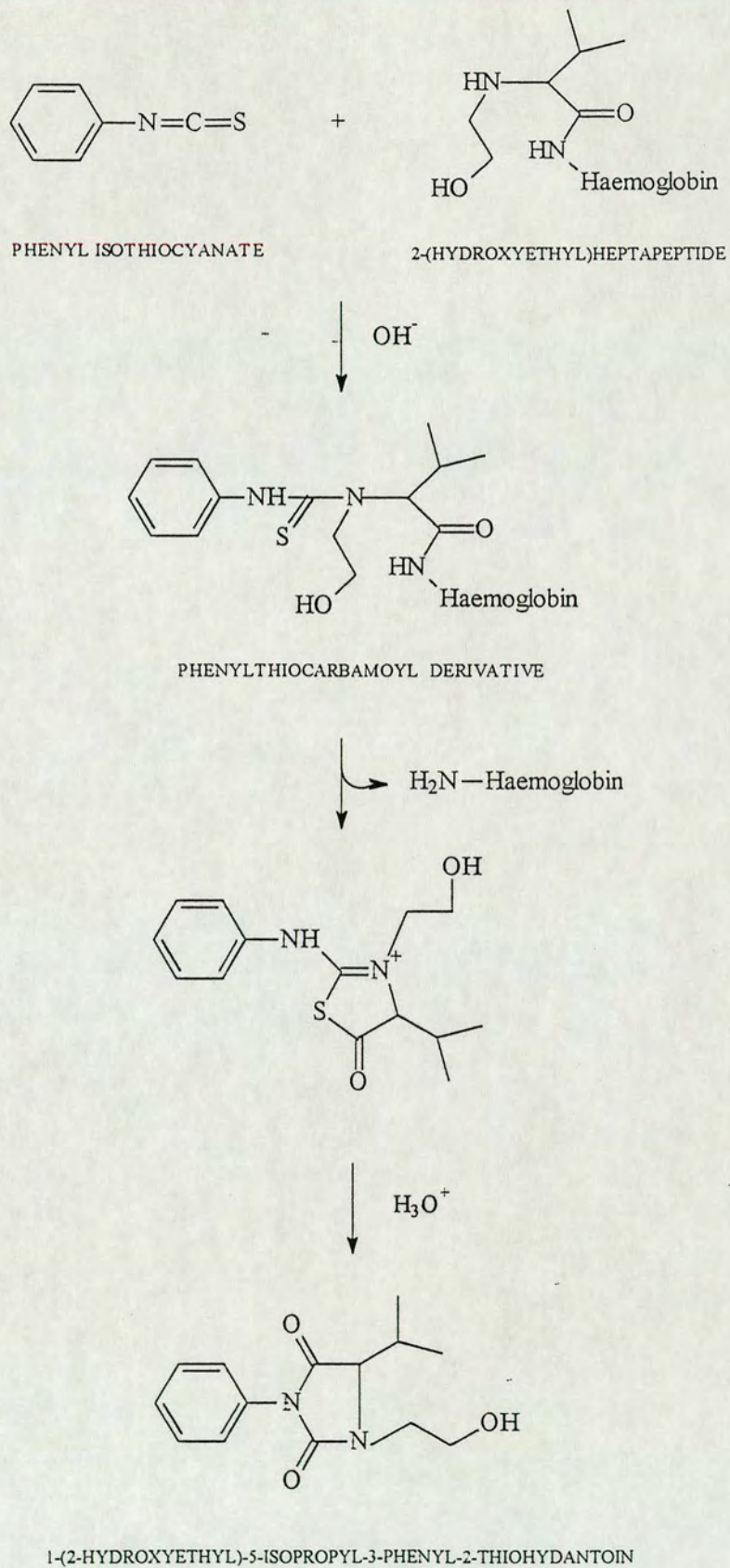
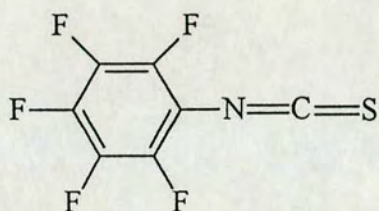
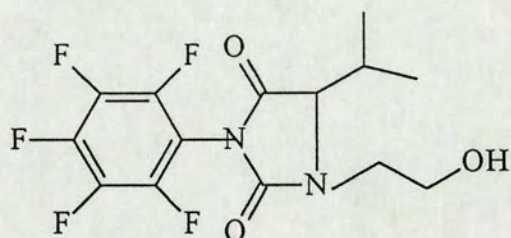


Figure 5.1 The Edman degradation reaction



PENTAFLUOROPHENYL ISOTHIOCYANATE (PFPITC)



1-(2-HYDROXYETHYL)-5-ISOPROPYL-3-PENTAFLUORO-PHENYL-2-THIOHYDANTOIN

Figure 5.2 The derivatisation agent PFPITC used in the modified Edman degradation procedure and the PFPTH adduct monitored by GC/MS

In addition to its high sensitivity, the Modified Edman degradation reaction has several advantages over early methods based on total protein hydrolysis:-

The method is 20-50 times faster.

Mild work up procedures reduce the risk of artefact formation during isolation of adducts from globin samples.

The method is adaptable to the determination of unknown adducts as fractionation of samples is not involved. All the substituted valines cleaved as PFPTH's and extracted, can be kept in one fraction to be subjected to GC/MS analysis.

However, a further increase in sensitivity is still desirable (Tornquist 1991). Furthermore, the method is taxing and involves a lengthy work-up prior to assay. Also, N-alkyl Edman Degradation - MS needs expensive equipment and can be carried out only in specialised laboratories.

(Kautiainen and Tornqvist (1990) reported a more generally applicable method determining hydroxyethyl and hydroxypropyl N-terminal valine adducts by GC analysis with ECD. Their results correlated excellently with the MS determinations but the pulsed ECD detector employed was significantly less sensitive. GC-ECD necessitates purification of the PFPTH's prior to analysis, which is carried out on Sep-Pak cartridges. The limit of detection of the ECD-GC method at present is 100 pmol/g globin which permits the detection of an increase in hydroxyethylvaline in smokers smoking 10 cigarettes a day, or an increment after exposures of 50ppb for ethylene oxide and 1ppm for ethene for 40 hours per week.)

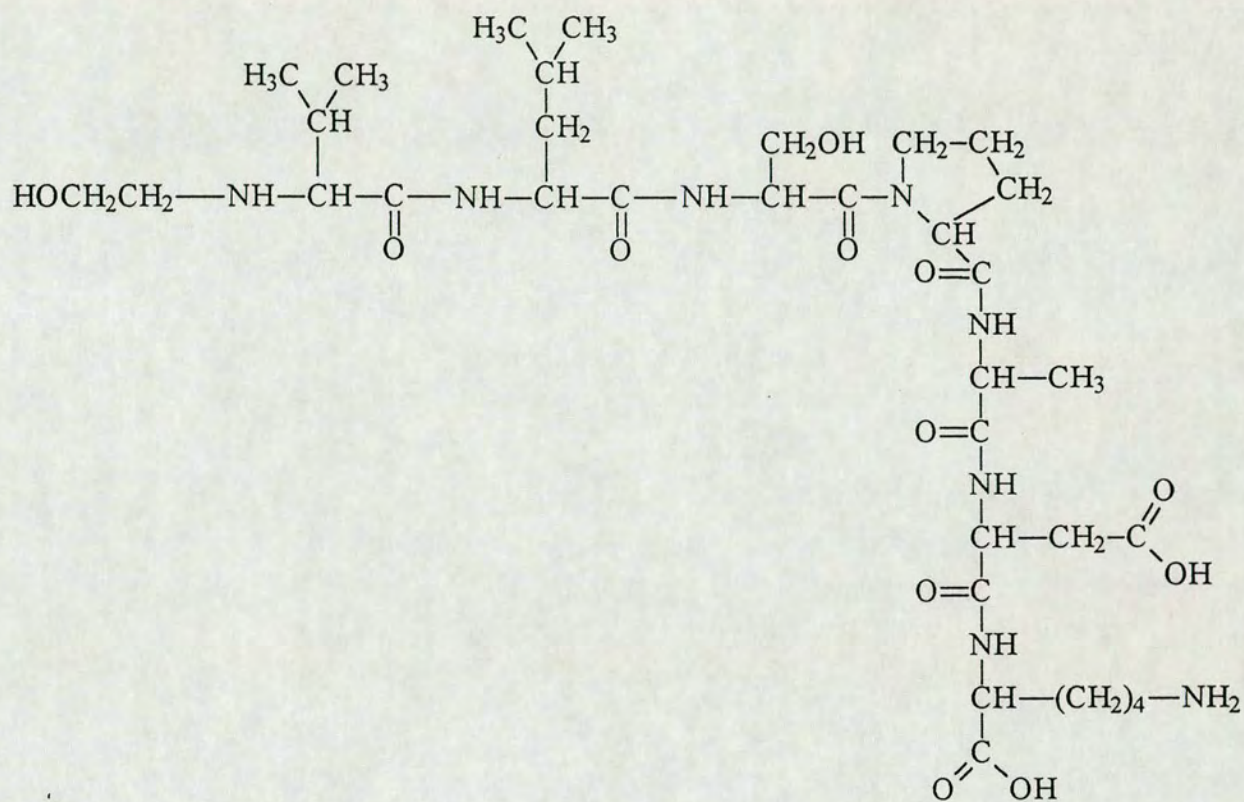
5.6 A RADIOIMMUNOASSAY FOR 2-(HYDROXYETHYL)HEPTAPEPTIDE

In this laboratory a specific and sensitive RIA has been developed for monitoring exposures to ethylene oxide and ethene (Wraith et al 1988). Polyclonal antibodies were raised to adducted N-terminal heptapeptides of the α chain of haemoglobin released from globin by the action of trypsin (fig. 5.3). The immunoassay could measure adducted heptapeptide in the presence of a 10^6 fold excess of non-adducted heptapeptide, and had a sensitivity of 0.14 pmol/g globin comparable to the GC-MS method described above. The RIA has been validated in a comparative study with the GC-MS method, and the results from samples analysed by the two procedures were in good agreement (correlation coefficient = 0.97).

Recently, the RIA has been converted to an ELISA. The ELISA has been used at the Shell Biomonitoring Laboratories and found to correlate well with the GC/MS procedure used there. However, high background values make the assay unsuitable for routine use (personal communication with H. Van der Waal, Shell Biomonitoring Laboratories). Polyclonal antibodies have a broad substrate specificity and problems associated with a high background may be overcome with a more specific monoclonal antibody.

5.7 IMMUNOAFFINITY CHROMATOGRAPHY

To determine adduct levels at low concentrations it is necessary to concentrate the adducts. The production of a monoclonal antibody which binds 2-(hydroxyethyl)heptapeptide will allow the development of immunoaffinity chromatography systems. Immunoconcentration may allow even lower adduct levels to be determined.



Hydroxyethylated Val-Leu-Ser-Pro-Ala-Asp-Lys

Figure 5.3 The N-terminal heptapeptide released from the alpha-chain of ethylene oxide treated haemoglobin by trypsin hydrolysis.

Concentration should be at the earliest stage and by the gentlest methods possible to avoid artefact formation. The optimum would be the quantification of adducted valines in whole haemoglobin or globin samples. The production of an immunoaffinity column which bound hydroxyethylated valine in whole haemoglobin or globin would greatly facilitate the assay of N-terminal adducted valines.

The aim of this aspect of the study was to produce a monoclonal antibody which recognises 2-(hydroxyethyl)heptapeptide in whole haemoglobin and to develop an immunoaffinity column to complement the GC/MS analysis of N-terminal adducted haemoglobin.

Due to the inaccessibility of the adducted N-terminal valines in whole haemoglobin adducted N-terminal heptapeptide from the alpha chain of haemoglobin was chosen for immunisation. The production of a monoclonal antibody which binds 2-(hydroxyethyl)heptapeptide, the development of an ELISA for measurement of 2-(hydroxyethyl)heptapeptide and antibody characterisation studies are described. Binding of adducted valine in whole haemoglobin is under investigation and the preliminary results are discussed. Finally, initial studies in to the development of an immunoaffinity procedure to facilitate biomonitoring studies is described.

METHODS AND MATERIALS

MATERIALS

REAGENTS

Ammonium hydrogen carbonate, haemoglobin, 2-(N-morpholino)-ethane sulphonic acid (MES) and trypsin type XIII (cat. no. T8642) were purchased from Sigma Chemical Co. Ltd. (Poole, Dorset. UK). Ammonium hydroxide, ethylacetate (analysed grade), ethyl ether (anhydrous, analysed grade) were purchased from J.T. Baker (Philipsburg, USA). Formamide (puriss), pentafluorophenyl isothiocyanate (PFPIITC, purim), n-pentane (UV spectroscopy) 2-propanol (p.A.) were purchased from Fluka Biochemika (Gillingham, Dorset. UK). Acetone (p.A), sodium carbonate (anhydrous, p.A.), sodium hydroxide (p.A.), toluene (p.A.) were available from B.D.H Merck (Merck Ltd., Dagenham, Essex. UK)

N-terminal hydroxyethylated heptapeptide and non-adducted heptapeptide was prepared by Cambridge Research Biochemicals Ltd. using solid phase peptide synthesis. After peptide synthesis the heptapeptides were analysed by HPLC and found to be homogeneous on two stationary phases. In addition, FAB-MS gave molecular weights consistent with those expected for the adducted (MW 772) and non-adducted heptapeptide (MW 728).

Hydroxyethylated heptapeptide coupled to horse serum albumin (HSA), hydroxypropyl adducted heptapeptide and whole adducted haemoglobin was supplied by Shell Research Ltd. (Sittingbourne, Kent. UK).

Adducted heptapeptide was coupled through its carboxyl and amino

functions of aspartic acid and lysine to amino and carboxyl groups on horse serum albumin (HSA) using 1-ethyl-3-(3-dimethylamino propyl)carbodiimide. Approximately 16 moles of adducted heptapeptide was conjugated per mole of HSA.

Hydroxypropyl adducted heptapeptide was released from whole haemoglobin after adduction with propylene oxide. The mono- and di-substituted heptapeptides were not separated.

Adducted haemoglobin prepared by the reaction of human haemoglobin with ^{14}C labelled ethylene oxide. From the incorporation of C^{14} it was estimated that each haemoglobin molecule contained at least one N-terminal modification.

BUFFERS

MES buffer consisted of 20mM 2-(N-morpholino)-ethanesulphonic acid pH 6.3

METHODS

5.8 PRODUCTION OF MONOCLONAL ANTIBODIES TO HYDROXYETHYL HEPTAPEPTIDE

IMMUNISATION PROTOCOL

Three female, 10-12 week old Balb/c mice were used for immunisation. Primary immunisations (day 1) and secondary immunisations (day 21) were given sub-cutaneously. Each mouse received 100ug of 2-(hydroxyethyl)heptapeptide conjugated to horse serum albumin in

200ul of a 50:50 v/v hapten adjuvant emulsion. Primary immunisations were prepared in Freund's complete adjuvant whilst secondary immunisations were prepared using Freund's incomplete adjuvant. Mice were bled (approx. 150ul) from the retro-orbital plexus (day 30).

Serum was separated from the blood samples by centrifugation (2200rpm for 5 minutes), and stored frozen at -70°C .

2. SCREENING ANTISERA

Antibody titre was determined by the preparation of antibody titration curves (1:30 to 1:7680). Relative affinity was determined by the preparation of standard calibration plots (1×10^{-11} to 1×10^{-9} moles/ml 2-(hydroxyethyl)heptapeptide) with a limiting concentration of antibody. Ovalbumin-2-(hydroxyethyl)heptapeptide (1ug/ml) and ethylene oxide derivatised haemoglobin (2.5ug/ml) were used for plate coating in the screening assays. Assay protocols are described in chapter 2.

3. CELL FUSIONS

Prior to fusion, mice were immunised with 100ug of immunogen in 100ul sterile PBS, intraperitoneally, on two consecutive days. Two days after the final pre-fusion immunisation, the mice were sacrificed by asphyxiation in diethyl-ether. The spleens were immediately removed from the mice and the spleen cells fused with the myeloma cell line Sp 2/0. Ag 1-4. as described in chapter 3. Two fusions were performed.

Fusions were screened by ELISA. Derivatised haemoglobin was used for plate coating (2.5ug/ml). Wells were recorded as positive in the screen with an O.D. of greater than two times background. Binding of free hapten was detected in binding inhibition studies with 500pmol/ml of 2-(hydroxyethyl)heptapeptide.

5.9 CHARACTERISATION OF MONOCLONAL ANTIBODY 4D3

Optimum plate coating concentration and antibody dilution were determined by "chequer-board" assays. Antibody affinity and specificity was determined from calibration curves. Cross-reactivity studies were performed with non-adducted heptapeptide, hydroxyethylheptapeptide and hydroxypropylheptapeptide. Calibration curves in the range 1×10^{-6} to 1×10^{-12} pmol/ml were prepared in the cross-reactivity studies.

5.10 BINDING STUDIES WITH WHOLE HAEMOGLOBIN.

Binding of adducted heptapeptide in whole haemoglobin was investigated by the preparation of calibration curves (1×10^{-6} - 1×10^{-12} pmol/ml) of adducted haemoglobin, non-adducted haemoglobin and 2-(hydroxyethyl)heptapeptide. Calibration curves were prepared in PBS/Tween.

5.11 DETERMINATION OF ADDUCTED 2-(HYDROXYETHYL)HEPTAPEPTIDE IN WHOLE HAEMOGLOBIN BY ELISA AFTER TRYPSIN HYDROLYSIS

GLOBIN PREPARATION - METHOD 1

Globin was extracted from whole adducted and non-adducted haemoglobins as follows. Haemoglobin solution was added dropwise with vigorous stirring to 20ml of 1% 1M HCl in acetone at -20°C . A freezing mixture of 3:1 (w/w) crushed ice:sodium chloride was used to generate a -20°C bath. The precipitated globin was separated from the haem by centrifugation (1720g for 10 minutes at -20°C). The supernatant was discarded. To avoid irreversible denaturation of the globin it was essential that in subsequent manipulations the protein was kept at -20°C . The globin was dissolved in 1ml of distilled water and reprecipitated by adding dropwise to acid/acetone as above. After centrifugation the precipitate was washed once with acid/acetone and twice with acetone alone. In later manipulations the globin became finer and the centrifugation time had to be increased (up to 40 minutes in the final wash).

After the final wash, traces of acetone were evaporated, initially under a gentle stream of nitrogen, and finally by freeze drying.

TRYPSIN HYDROLYSIS

Adducted globin, non-adducted globin and non-adducted globin spiked with 2-(hydroxyethyl)heptapeptide were hydrolysed as follows. Globin (10mg) was dissolved in distilled water (3ml). To this solution was added 100ul of 2mg/ml aqueous trypsin solution, followed by 600ul of 0.5M ammonium hydrogen carbonate. After mixing, the final pH was

checked (should be pH 8.5 +/- 0.2), and the solutions were reacted at 37°C for 2 hours. The pH of the solutions were adjusted to 6.5 by the addition of 0.2M HCl and made up to a final volume of 5ml with distilled water. The core peptides and the trypsin were precipitated by heating at 98°C for 10 minutes. After cooling the solution was clarified by centrifugation (2400rpm, 20 minutes at room temperature). The clear supernatant was decanted and an accurate volume freeze dried.

ASSAY OF TRYPSIN HYDROLYSED GLOBIN

The concentration of hydroxyethyl heptapeptide was determined by ELISA. Calibration plots were developed with the monoclonal antibody designated 4D3. Freeze dried samples were resuspended in distilled water. Resuspending the samples in 300ul of water gives a ten fold increase in adduct concentration.

5.12 DETERMINATION OF ADDUCTED N-TERMINAL VALINES BY THE MODIFIED EDMAN DEGRADATION REACTION

GLOBIN PREPARATION - METHOD 2

Haemoglobin samples were thawed on the day of use and mixed to ensure homogeneity. An aliquot (2.5ml) of haemoglobin was added dropwise to a freshly prepared solution 50mM HCl in 2-propanol with continuous stirring. The haem was separated from the globin and removed by centrifugation at 4500g for 10 minutes at room temperature. The supernatant, containing the globin, was decanted and the dark brown residue discarded. Ethyl acetate (25ml) was added

to the supernatant and the supernatant ethyl acetate mixture was allowed to stand for 30 minutes at room temperature. The globin precipitates were collected by centrifugation at 2000g for 10 minutes at room temperature. The supernatant was carefully decanted and discarded. The residue was resuspended in a further 25ml of ethyl acetate and the precipitation step repeated as described above. The globin was then resuspended in 25ml of n-pentane and the washed globin was centrifuged at 4500g for 10 minutes at room temperature to remove any traces of water from the sample. The supernatant was discarded and the globin was dried in a desiccator under vacuum overnight.

DERIVATISATION OF GLOBIN SAMPLES

50mg of dry globin was accurately weighed and dissolved in 1.5ml of formamide in a screw capped tube. Calibration standards containing 0-1800pmol HOEtValine/g globin were prepared in formamide from ethylene oxide treated globin containing an assessed 18nmol/mg globin. To each sample and standard was added 50ul of an internal globin standard solution. Globin standard was prepared from globin treated with perdeuterated ethylene oxide containing approximately 2.5nmol $^2\text{H}_4$ OEtValine/mg globin. Internal globin standard solution consists of 400ug of internal standard globin and 5mg of carrier globin per ml of formamide. Addition of 50ul of this solution to 50mg of sample corresponds to 1000 pmol $^2\text{H}_4$ OEtValine/g globin. After addition of internal standard the pH of the samples and standards was adjusted to 6.3 by the addition of 1M NaOH. The derivatisation reagent PFPITC was added (7ul) to each tube, the

tubes were capped, mixed and left in the dark overnight at room temperature. In order to complete the derivatisation the samples were finally warmed at 45⁰C for 1.5 hours.

CLEAN-UP OF SAMPLES

Deionised water (1ml) was added to each sample and the sample was extracted with 2ml of ethyl ether. After separation a further 1ml of deionised water was added and the sample extracted a second time with 3ml of ethyl ether. The etherial phases were combined and the ether evaporated under a gentle stream of N₂ gas. The residue was redissolved in 1ml of toluene.

The toluene solution was extracted with 2ml of deionised water to remove any traces of formamide. The toluene solutions were extracted twice with 2ml 0.1M aqueous sodium carbonate solution to hydrolyse any by-products. The toluene was extracted again with 2ml of water, the aqueous phase was separated from the toluene by brief centrifugation and discarded. Finally the toluene extract was evaporated to dryness at 50⁰C in a water bath under a gentle stream of N₂. The residue was redissolved in 50ul of toluene and analysed by GC/MS.

GC/MS ANALYSIS OF DERIVATISED 2-(HYDROXYETHYL)VALINE

GC/MS analysis was performed by the Department of Analytical Chemistry, Shell Research Ltd., Sittingbourne. Briefly, separation was achieved with a fused silica capillary column (30mx0.33mm, DB5 - Durabond) on a Finnigan Mat90 GC. The sample was loaded in toluene

and methane was used as the mobile phase. Detection was by mass spectrometry with selective ion monitoring (m/z 348 and 352). Methane was used as the reagent gas.

5.13 ANTIBODY PURIFICATION AND CONCENTRATION

Cation exchange chromatography:-

Cation exchange HPLC was performed with a Hydropore SCX column (10cm x 4.6mm). Before use the column was equilibrated with 20mM MES pH 6.3. Antibody solutions were diluted in MES buffer and filtered through a 0.2 μ m low protein binding membrane. Antibodies (10ml) were loaded onto the column at a flow rate of 1ml/minute, and the column washed with 15ml of MES buffer. Antibody was eluted with a gradient of 0-0.35M NaCl in 20mM MES pH 6.3 developed at 2ml/min over 10 minutes. Elution of protein from the column was monitored spectrophotometrically at 280nm. Fractions (2ml) were collected, and antibody activity was determined by immunoassay (preparation of antibody titration curves).

Protein G - affinity chromatography:-

Purification of antibody by cation exchange chromatography resulted in a loss in antibody binding. Therefore, antibody was also purified by protein G affinity chromatography and concentrated by membrane filtration. Membrane filtration was performed with an Amicon 30 centriprep filtration unit. Affinity purification and membrane filtration is described in 4.8 and 4.9 (pages 171-172).

5.14 PREPARATION OF AN IMMUNOAFFINITY COLUMN

COUPLING ANTIBODY TO CNBR ACTIVATED SEPHAROSE 4B

Antibody was coupled to CNBR activated sepharose (4.10, page 172). For coupling, concentrated antibody (1.5ml) was diluted in coupling buffer (1.5ml). Antibody uptake was determined from absorbance readings at 280nm.

5.15 AFFINITY CHROMATOGRAPHY

Before use, the immunoaffinity chromatography column was allowed to reach room temperature and equilibrated with PBS. Samples were chromatographed at a flow rate of approximately 0.5ml/minute.

2-(hydroxyethyl)heptapeptide was diluted in PBA (1ug/ml) and loaded (1ml) onto the immunoaffinity column. The column was washed with PBS (5ml) and eluted with methanol (2ml). Fractions, load (1ml), wash (1ml) and retentate (1ml) were collected. Methanol was removed by evaporation under a gentle stream of nitrogen. After evaporation the volumes were made up to 1ml by adding PBS. All the fractions were assayed for the presence of 2-(hydroxyethyl)heptapeptide by ELISA (3.91-1000ng/ml calibration curves were prepared).

RESULTS

5.16 MONOCLONAL ANTIBODY PRODUCTION

SCREENING ASSAY

Mice were bled 14 days after the second immunisations, yielding 50-150 μ l of serum per mouse. All the sera (x3) contained antibodies which bound both the ethylene oxide treated haemoglobin and adducted heptapeptide-ovalbumin conjugate coated plates in the screening assays. The antibody titre varied between the mice. The highest titres were observed with the derivatised haemoglobin coated plates (Table 5.1). All the sera contained antibodies which bound free 2-(hydroxyethyl)heptapeptide (Table 5.1). Binding to whole adducted haemoglobin was inhibited with 2-(hydroxyethyl)heptapeptide. The sera did not cross-react with non-adducted heptapeptide at 1000 pmol ml⁻¹.

Table 5.1 Screening results for 2-(hydroxyethyl)heptapeptide-ovalbumin immunised mice.

MOUSE	TITRE		INHIBITION*	
	Oval-Hb-OH	dHb	Oval-Hb-OH	dHb
1	1:100	1:300	36%	35%
2	1:100	1:300	17%	27%
3	1:300	1:400	25%	22%

* inhibition studies were performed with 500pmol/ml of 2-(hydroxyethyl)heptapeptide

Fusion 1

Spleen cells from mouse 1, immunised with hydroxyethylated heptapeptide conjugated to HSA, were successfully fused with the myeloma cell line Sp 2/O. Ag 1-4. On the day of screening 50-100% of the wells per plate contained 1 or more viable hybrid colonies. In the ELISA screen 37 supernatants were positive. No supernatants contained antibody which bound free hapten in the binding inhibition studies.

Fusion 2

Spleen cells from mouse 2 were successfully fused with the Sp 2/O. Ag 1-4 myeloma cell line. The hybrid colonies grew very rapidly and the fusion was screened after 7 days. On the day of screening all of the wells on the fusion plates contained 1 or more viable clones. The supernatants were screened by ELISA and 24 positive wells were obtained. Inhibition studies showed 6 supernatants contained antibodies which bound free hydroxethylheptapeptide ($500 \text{ pmol}^{-1}\text{ml}^{-1}$ 2-(hydroxyethyl)heptapeptide). The fusion plates were screened a second time, 7 days later, for slower growing hybridoma clones. 34 new positive wells were detected, none of which contained antibodies which bound free hapten.

Wells 1C8, 4D3, and 5F2, demonstrated the greatest inhibition in the binding inhibition studies (81%, 57% and 77% respectively). However, 1C8 supernatant also bound the non-adducted heptapeptide in the cross-reactivity studies and no further work was therefore carried out on this cell line.

Wells 4D3 and 5F2 were cloned and expanded as described in the methods. The results are summarized in table 5.2. Monoclonality was confirmed for well 4D3F3.

Table 5.2 Fusion 2 clone plate results

CLONED WELL	CLONE PLATE RESULTS		
	NOS. WELLS CONTAINING HYBRIDOMAS	NOS. +VE WELLS IN SCREENING ASSAY	NOS. MONOCLONAL WELLS +VE IN SCREENING ASSAY
4D3	79	12	5
5F2	84	0	0

5.17 CHARACTERISATION OF MONOCLONAL ANTIBODY 4D3

Assay conditions were optimised in "chequer-board" assay studies. The most sensitive assay conditions were achieved with 1:100 culture supernatant and either 0.25ug/ml 2-(hydroxyethyl)heptapeptide-ovalbumin or 1ug/ml adducted haemoglobin coated plates. Under these conditions 2-(hydroxyethyl)heptapeptide could be measured in the range 2×10^{-12} to 1×10^{-9} moles/ml (figure 5.4). The limit of detection was the same with both coating materials.

The antibody did not cross-react with non-adducted heptapeptide or hydroxypropylheptapeptide in the measuring range of the assay. Cross-reactivity did occur when non-adducted heptapeptide and hydroxypropyl heptapeptide were present in a 1250 fold excess. IC50 values for hydroxyethylheptapeptide, non-adducted heptapeptide and hydroxypropylheptapeptide were 8×10^{-11} , 1×10^{-7} and 1×10^{-7} moles/ml respectively (figure 5.5).

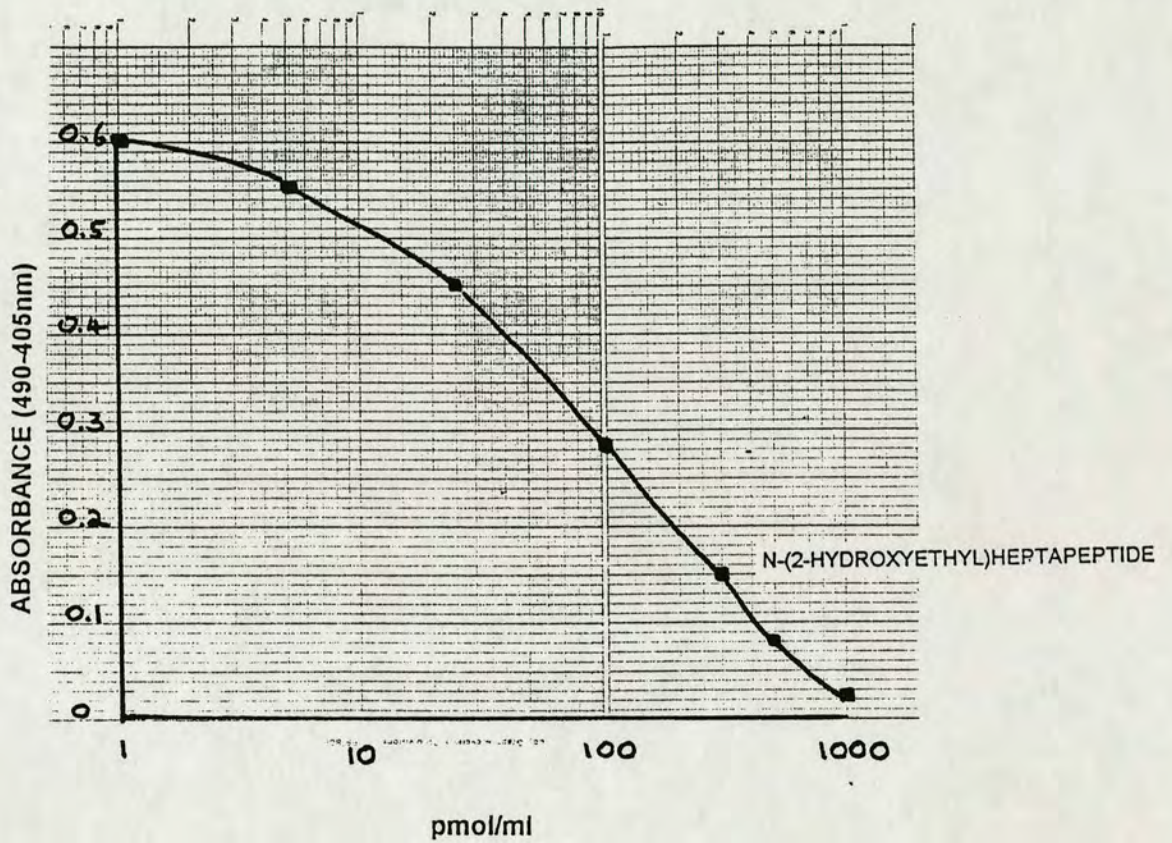


Figure 5.4 2-(hydroxyethyl)heptapeptide calibration plot developed with 4D3 in microtitre plates coated with derivatised whole haemoglobin

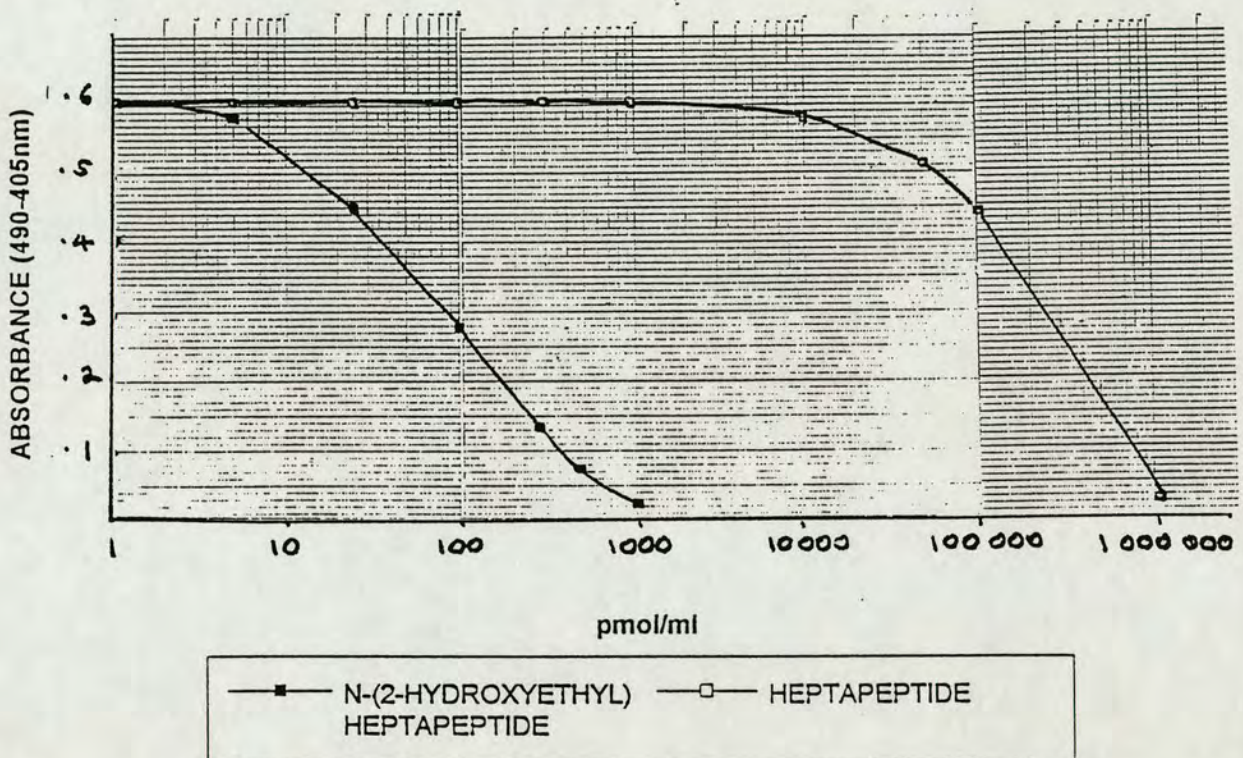


Figure 5.5 Cross-reactivity studies with 4D3. The ELISA was performed in plates coated with whole derivitised haemoglobin

5.18 BINDING OF 2-(HYDROXYETHYL)HEPTAPEPTIDE IN WHOLE HAEMOGLOBIN

Calibration curves were prepared with whole adducted haemoglobin, non-adducted haemoglobin and hydroxyethylheptapeptide. The antibody was able to distinguish adducted and non-adducted haemoglobin in the ELISA (figure 5.6). Assuming each haemoglobin molecule contains one adducted N-terminal valine then binding to whole haemoglobin was observed in the range 2×10^{-8} to 5×10^{-10} moles/ml of haemoglobin. This binding is approximately 0.6% of that expected. (IC50 values obtained with adducted haemoglobin and adducted heptapeptide were 7×10^{-11} and 3×10^{-9} moles/ml respectively).

5.19 DETERMINATION OF ADDUCTED 2-(HYDROXYETHYL)HEPTAPEPTIDE IN WHOLE HAEMOGLOBIN BY TRYPSIN HYDROLYSIS

56mg of globin was extracted from 10ml of 10mg/ml solution of adducted haemoglobin. After freeze drying the globin appeared as a white-pale brown solid. The N-terminal heptapeptide was released from the globin by trypsin hydrolysis. No adducted heptapeptides were detected in the control (non-adducted) haemoglobin. 90% of the 2-(hydroxyethyl)heptapeptide added to a non-adducted globin was measured in the ELISA. The adducted haemoglobin contained 25.2pmol/ml of adducted heptapeptide. After correction for loss of sample during preparation this corresponds to 28000pmol of adducted heptapeptide per gram globin. This was 0.2% of the expected value if each haemoglobin molecule contains one adducted terminal valine.

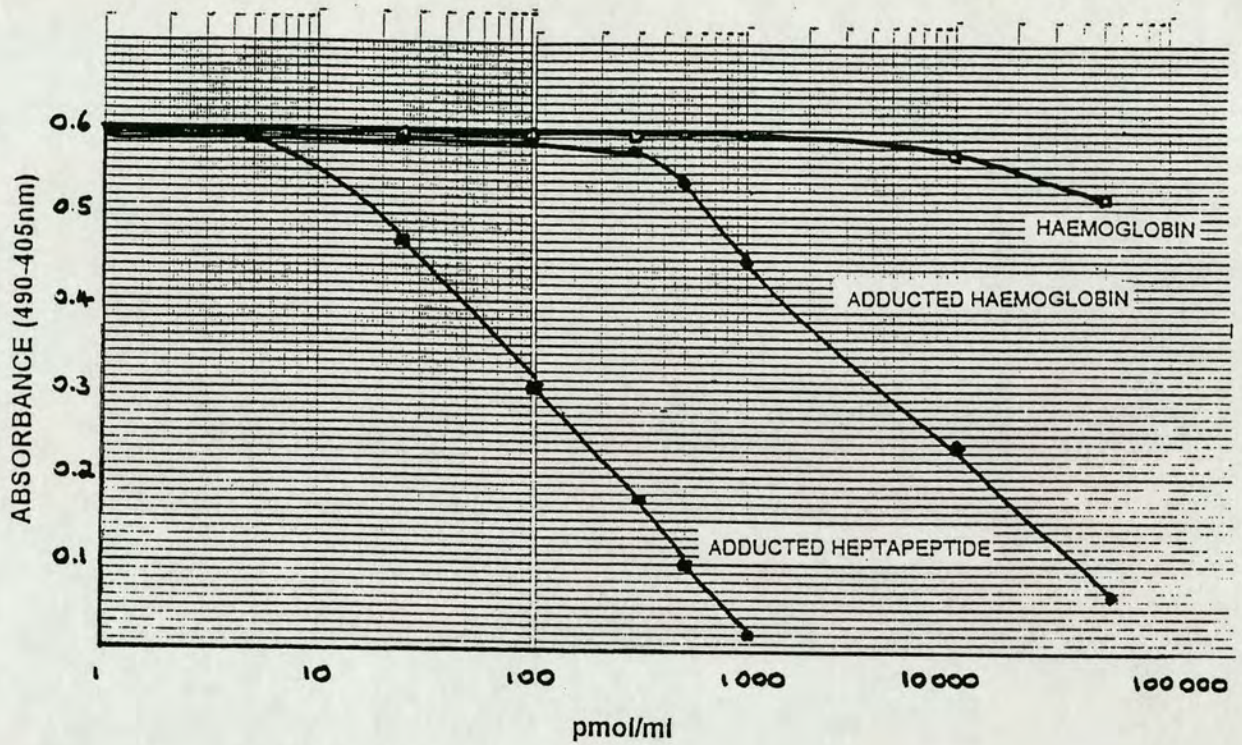


Figure 5.6 Adducted whole haemoglobin and adducted heptapeptide calibration plots developed with 4D3. Plates were coated with whole adducted haemoglobin.

5.20 DETERMINATION OF ADDUCTED 2-(HYDROXYETHYL)VALINE IN WHOLE HAEMOGLOBIN BY GC/MS (Modified Edman Degradation)

The adducted haemoglobin showed an increase in 2-(hydroxyethyl)heptapeptide from 70pmol/g globin in the background sample to 26745 pmol/g globin. The level of C14 equivalent peak at m/z 350 remains constant at 17-19%. It appears that the amount of C14 cannot be quantified at this level of activity.

5.21 PROTEIN PURIFICATION AND CONCENTRATION

Salt fractionation and cation exchange chromatography yielded 17mg of antibody from 500ml of cell culture supernatant (34ug/ml supernatant). A loss in antibody immunoreactivity was observed in binding studies after HPLC purification (table 5.3).

Protein G purification yielded 11.79mg of antibody (23.5ug of antibody per ml of culture supernatant). Antibody remained immunoreactive after protein G purification. The antibody titration curves demonstrate an 8 fold increase in 4D3 concentration. Antibody was concentrated a further 20 fold by membrane filtration. After membrane filtration 90% of the immunoglobulin was recovered (6.1mg/ml).

Table 5.3 Immunoreactivity (IC50 values) during antibody purification.

FRACTION	TITRE	
	HPLC PURIFIED	PROTEIN G PURIFIED
Culture supernatant	1:500	1:400
Purified antibody	1:60	1:3000
Concentrated antibody	1:300	1:60000

COUPLING TO SOLID PHASE

7.78mg (85%) of 4D3 was coupled to 0.38g (1.33ml of swollen gel) of CNBr activated sepharose. No protein was detected in the final high and low pH washes indicating that the antibody was covalently bound to the solid support.

5.22 IMMUNOAFFINITY CHROMATOGRAPHY

The immunoaffinity column had a maximum binding capacity of 4.7ug of hydroxyethylheptapeptide (6.6×10^{-9} moles). No hydroxyethyl heptapeptide was observed in the final washes when the fractions were analysed by ELISA.

DISCUSSION

Three mice were immunised with 2-(hydroxyethyl)heptapeptide conjugated to HSA. Antibodies which bind free hapten were induced in all the mice (table 5.1). Sera from mice 1 and 3 demonstrated the highest titres and relative affinity in the screening assay. Spleen cells from these two mice were successfully fused with the Sp2 myeloma cell line.

Six supernatants containing antibody which bound free hapten were detected when fusion 2 was screened by ELISA. Supernatant from well 4D3 demonstrated the greatest relative affinity and specificity for 2-(hydroxyethyl)heptapeptide. Well 4D3 was successfully cloned. The monoclonal cell line was cultured in stationary flasks and the supernatant harvested at regular intervals.

An ELISA has been developed with 4D3 which has a limit of detection of 2pmol/ml of adducted heptapeptide (Figure 5.4). The assay appears to be specific for 2-(hydroxyethyl)heptapeptide. Cross-reactivity with hydroxypropyl heptapeptide and non-adducted heptapeptide was observed only when present in a 1250 fold excess. The monoclonal assay should permit the quantification of approximately 0.6pmol of adducted N-terminal valine per gram of globin.

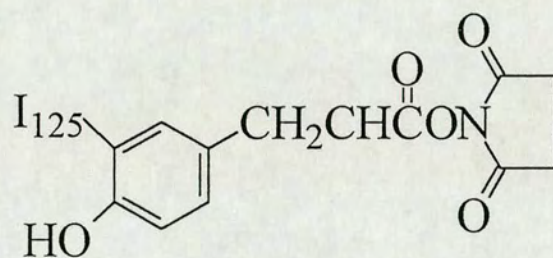
The ELISA developed with 4D3 is less sensitive than the assay of Wraith et al (1988) developed with a polyclonal antisera (sensitivity 0.14pmol/g globin). However the sensitivity compares favourably with the sensitivity of the GC/MS modified Edman Degradation procedure (sensitivity 1-10pmol/g globin). The

sensitivity of the ELISA is suitable for biomonitoring and it should be possible to determine the level of background adducts in man (0.3nmol/g globin, Tornqvist 1986). However, assay performance is yet to be validated with an alternative analytical procedure. This is essential if the utility of the monoclonal based assay is to be discussed. Validation will demonstrate if the problems associated with the polyclonal assay, high background and low adduct values at high concentrations, can be overcome with 4D3.

It is advantageous to detect antibodies which bind free hapten at the earliest possible moment after fusion. The first fusion was therefore, also screened by RIA. For the RIA 2-(hydroxyethyl)heptapeptide was labelled with ^{125}I using Bolton and Hunter's reagent (Figure 5.9). No antibodies which bound free hapten were detected with RIA, confirming the results of the ELISA.

Radiolabelled assays have a number of disadvantages. The stability of the isotope may limit the shelf life of the reagent. ^{125}I has a short half-life (64 days) and the labelling therefore must be repeated at regular intervals. Radioactivity is hazardous and radioactive waste must be disposed of. As a result, an alternative labelling strategy was investigated.

The use of a chemiluminescent label attached to an antibody has enabled the development high sensitivity assay systems (Collins 1985). In a novel approach, 2-(hydroxyethyl)heptapeptide was chemilumilabelled with the acridinium ester 4-(2-succinimidyl-oxycarbonylethyl)phenyl-10-methylacridinium-9-carboxylate (figure 5.10). Acridinium esters can be stimulated to produce light in the



N-SUCCINIMIDYL 3-(4-HYDROXY-5-[125I]IODOPHENYL)PROPIONATE

Figure 5.9 Bolton and Hunter's Reagent

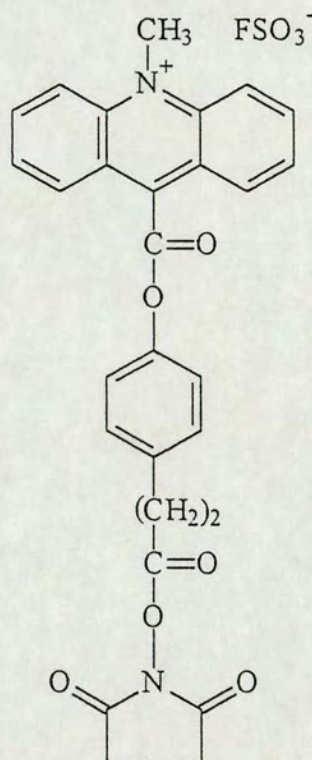


Figure 5.10 The acridinium ester 4-(2-succinimidylloxycarbonyl)phenyl-10-methylacridinium-9-carboxylate

in the presence of dilute hydrogen peroxide. The chemiluminescent reaction involves hydrolysis of the ester bond by the hydroperoxide ion and the formation of the electronically excited N-methylacridone. Consequently, the chemiluminescent moiety is released from the hapten prior to excitation. This reduces the quenching of labels associated with proteins, leading to a relatively high quantum yield. Proteins can be labelled with high specific activity and chemiluminescence has the potential of increasing assay sensitivity. In addition, chemiluminescent immunoassays can be readily converted into assay kits.

The integrity of the lumilabelled heptapeptide was demonstrated by the development of a calibration plot (2-250pmoles/ml) with the polyclonal antisera of Wraith (1988). At present this assay is five times less sensitive than the RIA/ELISA developed with the polyclonal antisera. The low sensitivity of the assay was due, in part, to the sensitivity of the detector which necessitated the use of high concentrations of label and antibody. Furthermore, when compared to the ELISA as a screening assay after fusion it was difficult to distinguish positive and negative supernatants due to high levels of non-specific binding. Although the assay remains to be optimised the application of this labelling procedure to 2-(hydroxyethyl) heptapeptide has been demonstrated. If the monoclonal based assay is successfully validated the development of a chemiluminescent assay, with a potential for improved assay sensitivity, will be investigated.

The recognition of adducted N-terminal valines in whole haemoglobin

was investigated. In initial studies comparable results were obtained when sera were screened against ovalbumin-adducted heptapeptide and ethylene oxide adducted haemoglobin coated plates. Also, no difference was observed between 2-(hydroxyethyl)heptapeptide calibration plots developed against adducted heptapeptide and ovalbumin heptapeptide coated plates. Next, 2-(hydroxyethyl)heptapeptide, adducted and non-adducted haemoglobin calibration plots were developed with 4D3. The antibody could distinguish between adducted and non-adducted haemoglobin but the concentration of adduct was less than expected (approximately 0.6%) assuming every haemoglobin molecule contains at least one adducted N-terminal valine.

The number of adducted N-terminal valines in whole haemoglobin was estimated from the incorporation of radioactivity after the reaction of haemoglobin with radiolabelled ethylene oxide. The number of adducted N-terminal valines was therefore determined more accurately. Determination of the concentration of adducted heptapeptide by ELISA after release by trypsin hydrolysis from whole globin found only 28nmol of adduct per gram of globin. This was 0.2% of the expected value. To confirm this result the number of 2-(hydroxyethyl)valines was determined using the modified Edman Degradation reaction and GC/MS analysis. The concentration determined by GC/MS analysis (27nmol/g globin) confirms the value obtained by ELISA. This data strongly suggests that 4D3 is able to quantitatively bind N-terminal 2-(hydroxyethyl)valine whole in adducted haemoglobin.

The aim of this part of the investigation was to develop an

immunoenrichment method to complement the Edman degradation procedure. The addition of an effective immunoenrichment to enrich whole adducted haemoglobin may reduce background interferences thereby enhancing the sensitivity of detection. It has been demonstrated that 4D3 retains its immunoreactivity after coupling to a solid support (CNBr activated sepharose) enabling the production of an immunoaffinity column. Immunoreactivity was assessed by monitoring binding of adducted heptapeptide applied in aqueous buffer. The column adsorbed 4.7ug of 2-(hydroxyethyl)heptapeptide and the analyte did not appear to leach from the column during washing. The column maintained its functionality after elution with solvent (95% methanol:water) allowing the same column to be used repeatedly. Purification of N-terminal adducted haemoglobin by immunoaffinity chromatography will now be examined.

CHAPTER SIX

FINAL DISCUSSION

The major aim of this project was to generate monoclonal antibodies which recognize mercapturic acids for application in the detection, identification and exposure monitoring of genotoxic carcinogens. This has been achieved.

A carefully designed hapten-protein conjugate and a judicious screening strategy has enabled the generation of a monoclonal antibody (3G9) which specifically binds S-PMA. Urinary S-PMA is a highly specific and sensitive marker of benzene exposure (Van Sittert et al 1993).

Benzene is an important industrial chemical and a group 1 carcinogen. Current occupational exposure limits (8 TWA) in the United Kingdom (MEL) and United States (PEL) are 5 and 1ppm respectively. Until now only GC/MS determination of S-PMA possessed the required sensitivity for application in biomonitoring programmes (0.3ppm 8 hours TWA, Boogaard and Van Sittert 1995).

Immobilized antibody retains immunoreactivity and can be used to enrich low levels of S-PMA (0.7-168ug/l) from the urine of benzene exposed workers. Bioconcentration of S-PMA from the urine of benzene exposed workers has permitted the quantification of S-PMA by HPLC at 8 hour TWA of less than 1ppm (background levels of urinary S-PMA can be detected). Furthermore, the performance of the immunoaffinity column has been validated against the solvent extraction GC/MS method of Boogaard (range 12-168ug/l, corr. coeff.=0.98, n=23).

Immunoaffinity chromatography/HPLC analysis is simple and easy to perform, unlike the GC/MS method which requires a complex work-up prior to assay and is restricted to use in sophisticated

laboratories. In addition, the sensitivity of the GC/MS procedure is limited by the purity of the sample. Immunoaffinity chromatography results in a cleaner sample than solvent extraction and as a result the sensitivity of the GC/MS analysis has been improved at least 10 fold.

Results obtained with 3G9 validate the immunochemical approach. The development of a rapid cost effective field assay to determine urinary S-PMA may improve human health exposure monitoring and permit more effective human health risk assessments. Immunoanalytical methods lend themselves to the development of field kits. The development of an antibody based test kit which will enable simple and rapid tests for benzene exposure to be carried out by operators in the field is now being investigated.

The development of simple methods to determine mercapturic acids will also have applications outside of industrial biomonitoring. Metabolism of benzene via benzene oxide leads to excretion of S-PMA in urine. Formation of benzene oxide is catalyzed by the cytochrome P450 system. Sex dependent differences and genetic polymorphism in the cytochrome P450 system exist and higher rates of metabolism may increase the susceptibility of individuals to benzene toxicity (Kenyon et al 1996). Similarly, phenotypic variation can also be found in the glutathione-S-transferases (GST's) and cancer susceptibility has been linked to phenotype. Polymorphisms may therefore have important implications in risk assessment. A simple immunoanalytical/HPLC method for the quantification of S-PMA should facilitate studies in this field.

In man 2-HEMA is a common urinary metabolite of a number of important industrial chemicals (eg. ethylene oxide, vinyl chloride and acrylonitrile). At present assays for 2-HEMA lack the desired sensitivity for application in biomonitoring programmes (Van Welie et al 1992). A second aim of this project therefore was to generate monoclonal antibodies which bind 2-HEMA for application in exposure monitoring programmes. 2-HEMA coupled to KLH was weakly antigenic and did not elicit a strong immunogen response. In a novel approach 2-HEMA was coupled to MSA. Using an homologous protein as the carrier molecule focused the immune response on the hapten. However the antibodies which we generated were generally of too low affinity for application in exposure monitoring studies.

Using a homologous protein carrier the practical possibility of producing class-specific antibodies to mercapturic acids has also been demonstrated. A low affinity, class-specific, antibody has been generated using acetylcysteine conjugated through its sulfhydryl group to MSA. Antibodies which bind mercapturic acids *per se* can be used to detect exposures to unknown genotoxic agents. Application of class-specific antibodies in an immunoextraction mode to concentrate unknown mercapturic acids should greatly facilitate the identification of unknown exposures using physico-chemical methods.

Antibodies which bound low molecular weight mercapturic acids formed a very small percentage of the total response. It would be expected therefore that the number of B lymphocytes secreting antibody which recognised these mercapturic acids will also form a very small percentage of the total. Cell fusion is inefficient, the genetic information of only about 1:10000 lymphocytes is fixed in the

hybridoma cells. Furthermore, hybridoma cells can be easily lost before monoclonality is reached. Using hybridoma technology the isolation of hybridoma cells secreting antibody which bound low molecular weight mercapturic acids has proved very difficult. Molecular biology techniques are increasingly been used to generate monoclonal antibodies (Owens and Young 1994). Using recombinant technology the entire gene repertoire is saved and expressed. Application of this technology should greatly increase the chances of isolating antibodies to small mercapturic acids. In addition, with mutagenesis it may be possible to alter the genetic framework of a low affinity antibody to give a high affinity antibody.

The framework of immunoglobulins has been used to generate recombinant libraries of proteins able to bind ligands of wide chemical diversity. Napolitano et al (1995) have demonstrated that the GST framework can also be used to generate a binding library. GST's are able to bind molecules of low molecular weight and this novel approach may generate proteins capable of binding low molecular weight compounds. Mercapturic acids may be suitable and exciting substrates for the development of novel binding proteins.

Reactive intermediates which gave rise to mercapturic acids may also give rise to protein conjugates. Haemoglobin adducts are commonly used in biomonitoring studies. We have demonstrated that monoclonal antibodies can be generated to adducted heptapeptide released from the alpha chain of haemoglobin by trypsin hydrolysis. To determine adduct levels at low concentrations it is necessary to concentrate the adducts. Concentration should be at the earliest possible stage and by the gentlest method possible to avoid artefact formation.

Initial studies strongly suggest antibodies can bind N-terminal adducted valines in whole haemoglobin. The development of immunoextraction procedures which recognize N-terminal valines in whole haemoglobin may facilitate the quantification of low adduct concentrations.

For the ultimate carcinogen to bind covalently to haemoglobin it must be sufficiently stable to diffuse out of the cell in which the carcinogen is metabolised and into an erythrocyte. This involves crossing two cell membranes. Serum proteins are more readily available and as a result adduct formation should be more efficient. In addition, serum proteins are synthesized in hepatocytes and it is in hepatocytes that many carcinogens are metabolised (Skipper 1990).

The benzene metabolite, benzene oxide, not only undergoes reaction with glutathione but may also react with cysteine groups in other protein molecules (Bechtold, 1992). Serum albumin of man contains only one cysteine molecule (at position 34) with a reactive sulfhydryl group which is known to bind xenobiotics. Bechtold has demonstrated a linear response between benzene exposure and S-phenylcysteine formation, and was able to measure S-phenylcysteine in occupationally exposed humans. After protein hydrolysis Bechtold determined trifluoroacetyl derivitised S-phenylcysteine by GC/MS. The method is time consuming and the sensitivity is limited by the purity of the sample. Antibody 3G9 binds S-phenyl-N-trifluoroacetylcysteine. Immunoaffinity chromatography has the potential to improve the sensitivity and facilitate the determination of this adduct in albumin. This should be investigated.

Determination of adducts in albumin, like haemoglobin, is generally laborious and time consuming. The determination of adducts in whole albumin may greatly facilitate biological monitoring and metabolism studies. These studies have shown that antibodies can be generated that bind adducted MSA but which show no cross-reactivity with non-adducted albumin. Furthermore, these antibodies demonstrate exquisite specificity, and are suitable for detecting low molecular weight (eg. hydroxyethylation) conjugates. Determination of albumin adducts formed by exposure to small molecular weight compounds (eg. butadiene and isoprene) may be rewarding.

Mercapturic acids are a record of exposure in the last 24 hours. Serum albumin and haemoglobin have half-lives of 20-25 days and 120 days respectively in man and are a record of chronic exposure. However, exposures over a life-time determine an individual's risk to cancer. Some proteins have very long life-times (eg. collagen) and may prove to be lifetime dosimeters (Skipper 1990). Antibodies are induced with one protein conjugate and screened against a second protein conjugate. Antibodies which recognize adducted proteins *per se* may have applications in this field of study.

In 1895 Wohler wrote *"Organic chemistry nowadays almost drives me mad. To me it appears like a primeval forest full of the most remarkable things, a dreadful endless jungle into which one does not dare enter for there seems no way out"*. Today that jungle still exists and remains largely unexplored. Although monoclonal antibodies give us one tool with which to travel, this study demonstrates, in part, how much more must still be mapped.

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APPENDIX

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Ball, R.L., Aston, J.P. and Van Sittert. N.J. (1995)
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