EFFECT OF AGED GARLIC EXTRACT ON THE HEPATOTOXICITY AND METABOLISM OF BROMOBENZENE IN RAT LIVER SLICES

A thesis submitted in partial fulfilment of the requirements of

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DECLARATION

I declare that the work presented in this thesis is all my own work and that it has not been submitted for any other degree.

BOHAN WANG

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I am forever grateful to my Mum and Dad for giving me a such wonderful life, constant love and good education. Thanks to Weiqun for sharing the life with me.

To my mother and father

ABSTRACT

Precision-cut rat liver slices (8 mm diameter, 250 μm thick) were produced with a Krumdieck tissue slicer and incubated individually in 24-well culture plates filled with 0.5 ml of Dulbecco's Modified Eagles medium, pH 7.4. The plates were incubated at 37 °C on an orbital shaker in an atmosphere of 95% air/5% CO_2. Several indicators of slice viability reflecting different aspects of cell function and integrity were determined. Thus, the release of total protein, alkaline phosphodiesterase (PDE), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH), slice K^+, ATP and non-protein sulphydryl (NPSH) content, rates of protein synthesis and secretion, and, slice ethoxycoumarin O-deethylase (ECOD) activity were all determined. Viability of cultured slices was well maintained over 24 h as judged by all parameters evaluated except ECOD activity which declined to 50% of its initial level after 8 h in culture.

The industrial solvent and model hepatotoxin bromobenzene (BB) is metabolised by cytochrome P-450 isoforms predominantly to the corresponding 3,4-oxide which, at low concentrations, is detoxified by conjugation to glutathione (GSH). At higher concentrations, GSH becomes depleted and the 3,4-oxide causes toxicity via binding to tissue proteins and/or mediating lipid peroxidation. Rats were treated with phenobarbital to induce cytochrome P-450 isoforms and potentiate BB toxicity then liver slices from these animals were incubated for up to 8 h with BB at final concentrations of 0.1 - 1 mM. Under these conditions, BB caused dose- and time-dependent cytotoxicity as judged by loss of intracellular K^+, ATP and NPSH, release of LDH, ALT and PDE, inhibition of protein synthesis and secretion, and histological evaluation.

The effects of aged garlic extract (AGE), S-allyl cysteine (SAC, the major organosulphur compound in AGE) and N-acetyl cysteine (NAC) on BB-induced toxicity were also examined using liver slices prepared from phenobarbital-induced rats. Addition of AGE at concentrations of 1-5% (v/v) to the culture medium reduced the toxicity of BB in a concentration-dependent manner as judged by slice K^+, ATP, NPSH and GSH content and the release of LDH and ALT into medium. AGE also inhibited lipid peroxidation when measured as the formation of thiobarbituric acid reacting substances (TBARS) which was reduced to control levels even at the lowest concentration of AGE. The BB-induced loss of slice K^+ and NPSH, release of LDH and ALT, and, TBARS formation were also prevented by the inclusion of up to 10 mM SAC or NAC in the culture medium. The protection against BB toxicity by SAC was similar to that observed by AGE suggesting that SAC is a key hepatoprotective compound in AGE. In addition, the protective effects of AGE, SAC and NAC on BB-induced toxicity still occurred in the presence of the GSH synthesis inhibitor buthionine sulphoximine suggesting that short term *de novo* GSH synthesis is not necessary for hepatoprotection. AGE and SAC were also found to cause partial inhibition of microsomal cytochrome P-450 when assayed as both ECOD and 7-pentoxyresorufin O-dependentylase (PROD) activities, but even the highest concentration used inhibited both activities by approximately 50%.

The effect of feeding AGE for 7 days to rats at doses of 2 and 10 ml/kg/day on BB toxicity to subsequently isolated liver slices was also examined. Pretreatment with AGE dramatically reduced the toxicity of BB in a dose-dependent manner. The GSH content of slices from rats treated with AGE was increased by 50-70% after 5 days whilst microsomal ECOD and PROD activities were not affected. Thus, the mechanism of the protective effect of AGE against BB hepatotoxicity involves both a GSH sparing effect (possibly due to conjugation of organosulphur compounds in AGE with toxic BB metabolites) and a long term elevation of GSH (see only after feeding AGE).

ABBREVIATIONS

AGE	Aged Garlic Extract
ALT	alanine aminotransferase
ATP	adenosine 5'-triphosphate
BB	bromobenzene
BSA	bovine serum albumin
BSO	L-buthionine-[S,R]-sulphox-imine
°C	degrees centigrade
Ci	curie
dH_2O	distilled deionised water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulphoxide
DPM	disintegrations per minute
DTNB	5,5'-dithio-bis(2-nitrobenzoic acid)
7-EC	7-ethoxycoumarin
7-ECOD	7-ethoxycoumarin O-deethylase
EDTA	ethylenediamine tetraacetic acid
FLE	firefly lantern extract
g	gram
G-6-P	glucose-6-phosphate
GSH	glutathione
GSSG	glutathione disulphide
h	hour
7-HC	7-hydroxycoumarin
IU	international unit
kD	kilo Daltons
LDH	L-lactate dehydrogenase
M	molar
MDA	malondialdehyde
mg	milligram
ml	millilitre

mM	millimolar
NAC	N-acetyl-L-cysteine
NAD	β-nicotinamide adenine dinucleotide
NADH	β-nicotinamide adenine dinucleotide (reduced form)
NADP	β-nicotinamide adenine dinucleotide phosphate
NADPH	β-nicotinamide adenine dinucleotide phosphate (reduced form)
nM	nanomolar
nmol	nanomoles
NPSH	non-protein sulphydryl
OD	optical density
P	level of statistical probability
P-450	cytochrome P-450 isozymes
PCA	perchloric acid
PDE	alkaline phosphodiesterase
PNPPT	thymidine 5'-monophosphate p-nitrophenyl ester
ppm	parts per million
PROD	7-pentoxyresorufin O-depentylase
RER	rough endoplasmic reticulum
rpm	revolutions per minute
SAC	S-allyl cysteine
SD	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-Polycrylamide gel electrophoresis
SER	smooth endoplasmic reticulum
TBA	2-thiobarbituric acid
TBARS	thiobarbituric acid reacting substances
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	Tris hydroxy-ethylamine
vs	versus
v/v	volume to volume

w/v	weight to volume
µl	microlitres
µM	micromolar
µmol	micromoles

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CHAPTER 1

INTRODUCTION

1.1 Toxic Response of the Liver

The liver has many metabolic roles and is affected by a very large number of xenobiotics. Liver injury induced by chemicals has been recognised as a toxicological problem for over 100 years (Zimmerman, 1978). The susceptibility of the liver to injury by chemical agents appears to be a consequence of its anatomical position and the central role it plays in the disposition and metabolism of xenobiotics.

1.1.1 Liver structure and function

The liver is a large organ making up about 3.5% of the body weight of an adult rat or 2% of the body weight of an adult human (Hinton and Grasso, 1993). Residing between the intestinal tract and the rest of the body, the liver is strategically positioned to perform its task of maintaining the body's metabolic homeostasis. The liver has a complex blood supply. Approximately 80% of the blood is derived from the hepatic portal vein. This drains the duodenum, small intestine and a portion of the colon. The remaining 20% of the blood supply comes from the hepatic artery. Blood leaves the liver by the very short hepatic veins which join the ascending vena cava. Venous blood from the stomach and intestines flows into the portal vein and then through the liver before entering the systemic circulation. In addition, the liver acts as an exocrine gland, secreting bile. This bile is conducted down the extrahepatic bile duct into the intestine.

Because of its circulatory architecture, the liver is the first organ to encounter ingested nutrients, vitamins, metals, drugs, and environmental toxins as well as the waste products of intestinal bacteria that enter the portal blood. Efficient scavenging or uptake processes extract these absorbed materials from the blood for catabolism, storage, and / or excretion into bile. The liver can be regarded as the central organ in metabolism and in metabolic regulation in that it maintains the continuous supply of energy substrates for the organism. The structural and functional units of the liver can be shown in Figure 1.1 and 1.2. The liver contains multiple cell types (Figure 1.1). Based on stereological analysis, the parenchymal cells, commonly referred to as "hepatocytes", are found to constitute 92.5% of the total volume of liver cells (Li, 1994). The remainder of the cells are sinusoidal and perisinusoidal cells, including the fenestrated endothelial cells, the stationary macrophage Kupffer cells, and the lipocytes. As shown in Figure 1.2, a small branch of the hepatic vein extends through the centre of each lobule. Around this central vein, in plates or irregular walls radiating outward, are arranged the hepatic cells. Around the periphery of each lobule, several sets of three tiny tubes-branches of the hepatic artery, of the portal vein (interlobular veins), and of the hepatic duct (interlobular bile ducts)-are arranged. Blood enters a lobule from branches of the hepatic artery and portal vein. Arterial blood oxygenates the hepatic cells, whereas blood from the portal system passes through the liver for "inspection". Sinusoids in the lobule are lined with reticuloendothelial cells (mainly Kupffer cells). These phagocytic cells can remove toxic materials from the bloodsteam. Ingested vitamins and other nutrients to be stored or metabolised by liver cells also enter the hepatic cell "bricks", forming radiating walls of the lobule. Bile formed by hepatic cells passes through canaliculi to the periphery of the lobule to join small bile ducts.

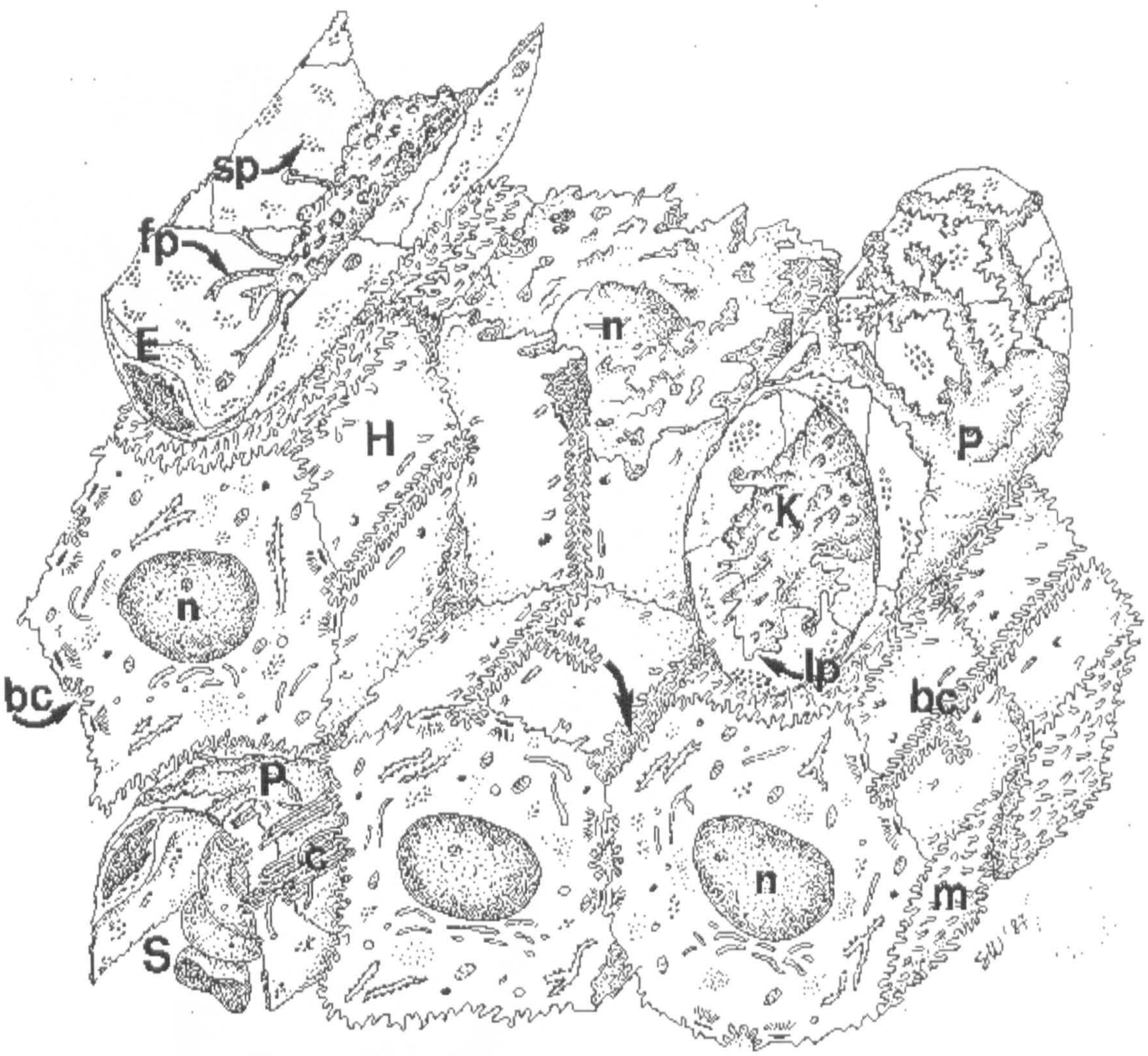


Figure 1.1 The liver cells. The diagram shows the three principal liver sinusoidal cells (endothelial cell-*E*, Kupffer cell-*K,* and perisinusoidal cell-*P*) and their spatial relationship to hepatocytes *(H),* as seen by scanning electron microscopy. Biliary facets are characterized by straight and centrally placed bile hemicanaliculi *(bc)* sided by smooth, microvilli-free areas, presenting studs and holes. Vascular facets are richly covered by microvilli *(m)* (the latter also extend in the interhepatocytic recesses-large curved arrow) and have close relationships with collagen-reticular fibers *(c)* and with the perisinusoidal cell processes. These processes, flat and branched, surround the sinusoids *(s)* determinng the second layer of the liver capillary wall. Endothelium is flat and provided with fenestrations, often clustered (sieve plates-*sp*) which may be crossed by the cytoplasmic prolongations of the liver macrophages. The latter, the Kupffer cells, lie on the sinusoidal endothelium and have very close relationship with the sinusoidal wall through their filopodia *(fp)* and lamellipodia *(lp). n,* nuclei. (From Tavoloni & Berk, 1993)

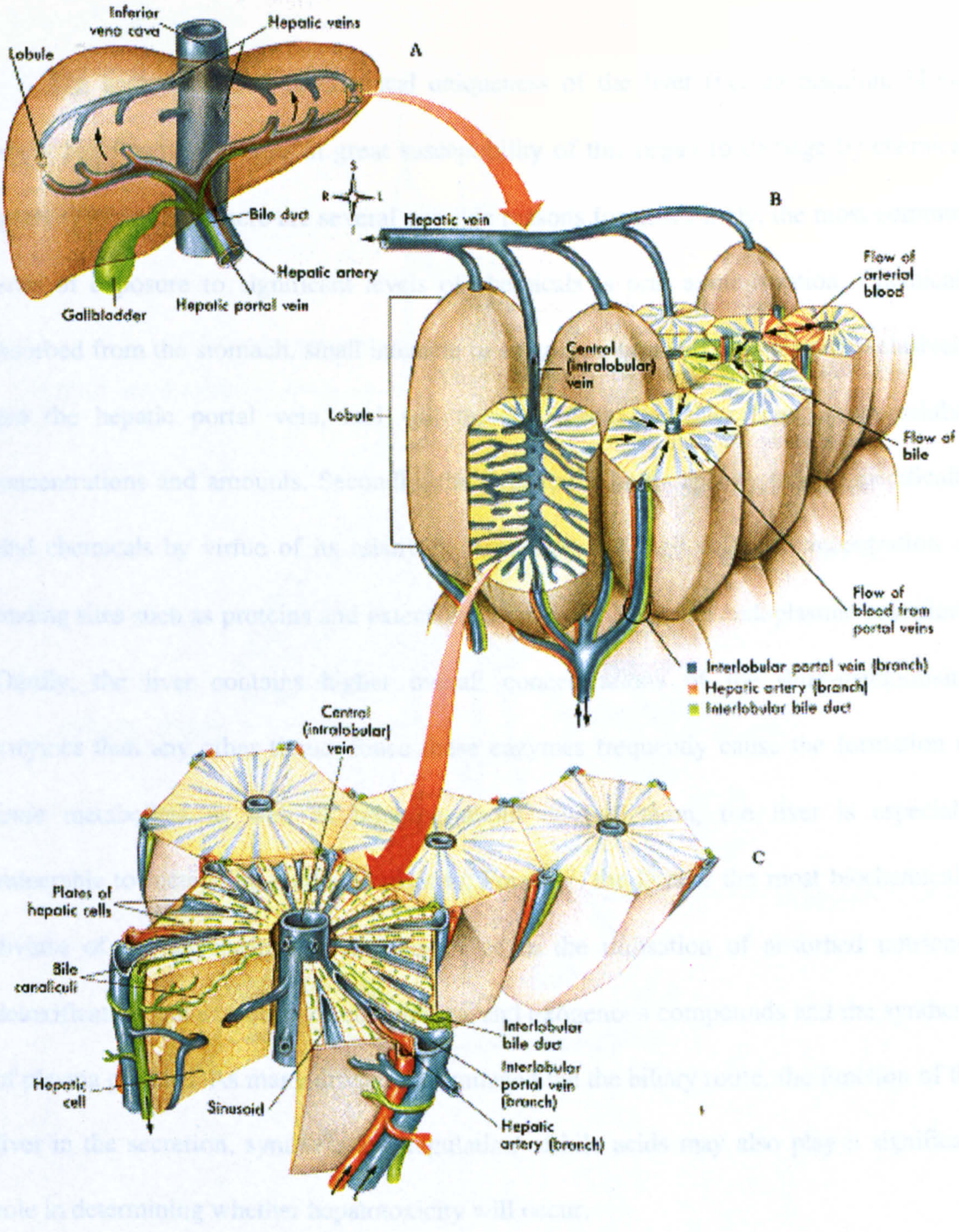


Figure 1.2 Microscopic structure of the liver. A, This diagram shows the location of the liver lobules relative to the overall circulatory scheme of the liver. **B** and **C,** Enlarged views of several lobules show how blood from the hepatic portal veins and hepatic arteries flows through sinuses and thus past plates of hepatic cells toward a central vein in each lobule. Hepatic cells form bile, which flows through bile canaliculi toward hepatic ducts that eventually drain the bile from the liver. (From Thibodean & Patton, 1993)

1.1.2 Liver injury

The anatomical and biochemical uniqueness of the liver (i.e. its position, blood supply and function) results in great susceptibility of this organ to damage by chemical agents (Figure 1.3). There are several possible reasons for this. Firstly, the most common route of exposure to significant levels of chemicals is oral administration. Chemicals absorbed from the stomach, small intestine or large intestine will pass almost exclusively into the hepatic portal vein, and will thereby reach the liver first in appreciable concentrations and amounts. Secondly, the liver has a high capacity to non-specifically bind chemicals by virtue of its relatively large size and high cellular concentration of binding sites such as proteins and extensive amounts of lipid-rich endoplasmic reticulum. Thirdly, the liver contains higher overall concentrations of the drug-metabolising enzymes than any other tissue. Since these enzymes frequently cause the formation of toxic metabolites as well as bringing about detoxification, the liver is especially vulnerable to metabolite-mediated toxicity. Fourthly, the liver is the most biochemically diverse of all the organs, playing key roles in the utilisation of absorbed nutrients, detoxification and excretion of endogenous and exogenous compounds and the synthesis of plasma proteins. As many drugs are eliminated via the biliary route, the function of the liver in the secretion, synthesis and regulation of bile acids may also play a significant role in determining whether hepatotoxicity will occur.

This great range of activities renders the liver more vulnerable to toxins than many other organs (Bridges, 1981). However, probably the most important factor accounting

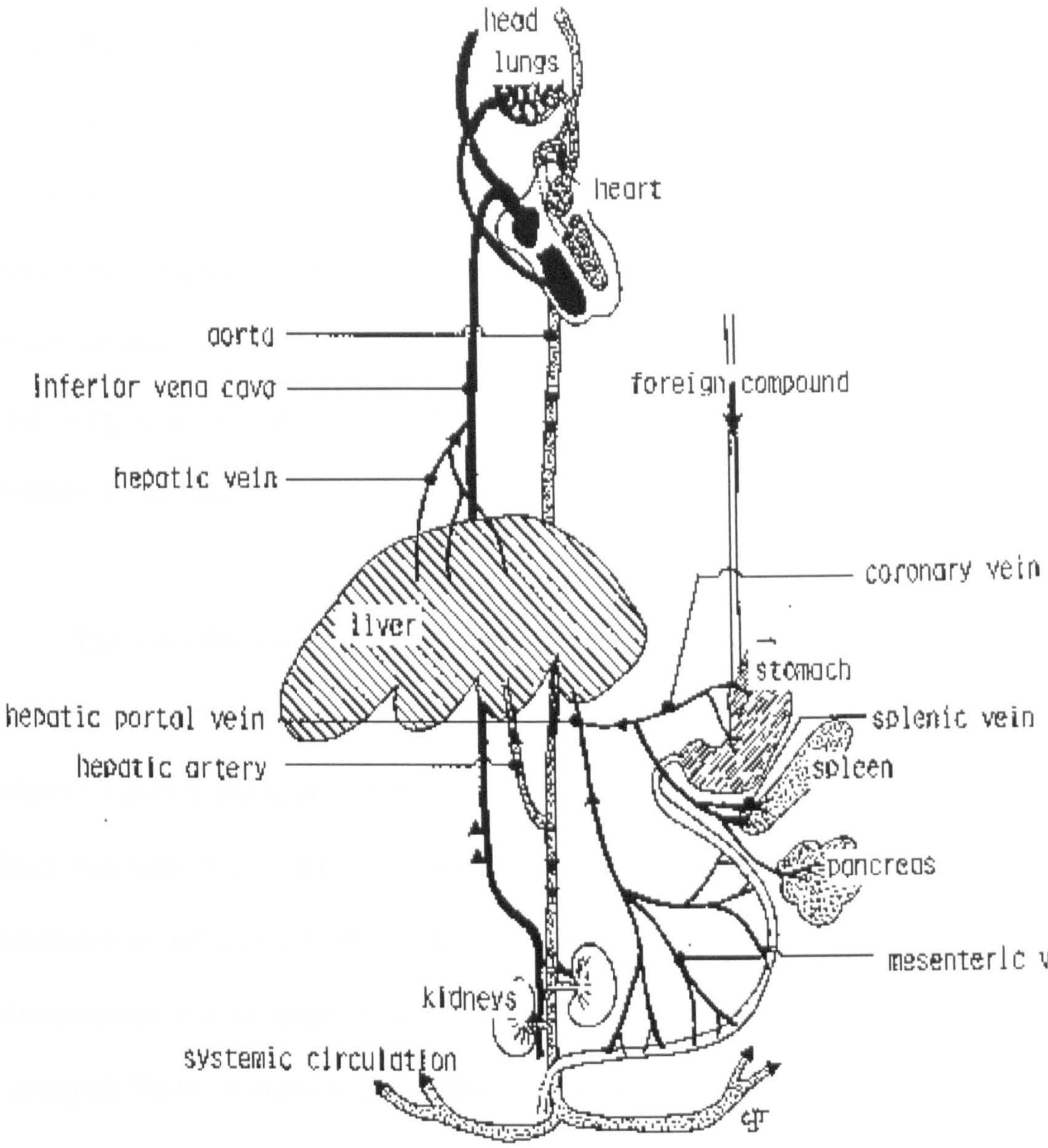


Figure 1.3 The vasculature supplying and draining the liver and its relationship to the systemic circulation. (From Timbrell, 1991)

for the susceptibility of the liver to chemical-induced toxicity is the major role this organ plays in the bioactivation of xenobiotics since a high biotransformation capacity is a critical factor for many xenobiotics, especially since hepatic biotransformation processes are readily modified by exposure to other chemicals. It has been recognised for many years that the liver is quantitatively the most important site of drug metabolism in the body containing the highest levels of cytochrome P-450 isozymes. As cytochrome P-450 is responsible for the bioactivation of many xenobiotics to toxic species it is not surprising to learn that in many instances biotransformation by the liver may result in toxicity to the organ.

The liver has only a limited number of responses to insult and thus different biochemical alterations may lead to the same toxic end-point. No single mechanism seems to govern the appearance of degenerative changes in hepatocytes or alterations in liver function. Some forms of liver injury are reversible while others result in a permanently unbalanced organ. As a result of large reserves in metabolic capacity disturbances in liver function may not always be detectable until the organ is severely damaged. Some common manifestations of liver injury are: necrosis, hepatitis, fatty liver (liposis), canalicular cholestasis, cirrhosis, bile duct damage, tumours (carcinogenesis) and hepatocyte death.

1.2 Xenobiotic Metabolism and Cytotoxicity

Most chemicals undergo metabolic transformations and there are numerous biochemical pathways involved. These biotransformations are usually associated with an

increase in the water-solubility of a drug, which in turn leads to an increase in the rate of renal and / or biliary excretion. The chemical changes involved usually result in a loss or diminution of pharmacological activity and drug metabolism is therefore generally considered to be a detoxification process. However, it should be emphasised that drug metabolism has additional, equally important roles to play in the toxicity of drugs; thus, a totally innocuous drug can be converted to a highly reactive metabolite which, by a variety of mechanisms, can result in a toxic response.

Drugs and xenobiotics are transformed by a variety of chemical pathways in two distinct stages (Williams, 1959). The phase I reactions involving oxidation, reduction and hydrolysis serve to introduce a suitable functional group into the drug molecule (-OH, -NH_2, -SH, -COOH etc.), thereby changing the drug in most cases into a more polar form, which is more water soluble than the parent molecule and hence is a more readily excretable form. However, the prime function of phase I metabolism is to permit the compound to undergo phase II reactions and not to prepare the drug for excretion. Phase II reactions are biosynthetic reactions where the foreign compound or a phase I - derived metabolite is covalently linked to an endogenous molecule producing a conjugate, thereby leading to increased water solubility and elimination from the body. Table 1.1 shows some reactions involved in phase I and phase II metabolism together with the enzymes catalysing their reactions.

In quantitative terms, the liver is the main organ responsible for phase I and phase II biotransformations although the kidney, adrenal cortex, lungs, placenta, skin, and even lymphocytes may be involved to a lesser extent.

Table 1.1 Enzymes and Reactions in Phase I and Phase II Metabolism

Phase I Reactions	**Enzymes**
Oxidation	
A. Microsomal oxidation	Cytochrome P-450
1. Aliphatic oxidation	
2. Aromatic hydroxylation	
3. Epoxidation	
4. Oxidative deamination	
5. N-, O-, S-dealkylation	
6. N-, P-oxidation	
7. N-hydroxylation	
8. Sulphoxidation	
9. Desulphuration	
B. Non-microsomal oxidations	
1. Amine oxidation	Amine oxidases
2. Alcohol dehydrogenation	Alcohol dehydrogenase
3. Aldehyde dehydrogenation	Aldehyde dehydrogenase
Reduction	NADPH-cytochrome c reductase
A. Microsomal reduction	
1. Nitro reduction	
2. Azo reduction	
B. Non-microsomal reduction	
Hydrolysis	Various esterases
Phase II Reductions	**Enzymes**
Glucuronide formation	UDP-Glucuronyl transferase
Sulphate conjugation	Sulphotransferase
Methylation	Methyl transferase
Acetylation	N-acetyl transferase
Amino acid conjugation	N-acyltransferase
Glutathione conjugation	Glutathione S-transferase

(Compiled from Lu, 1985)

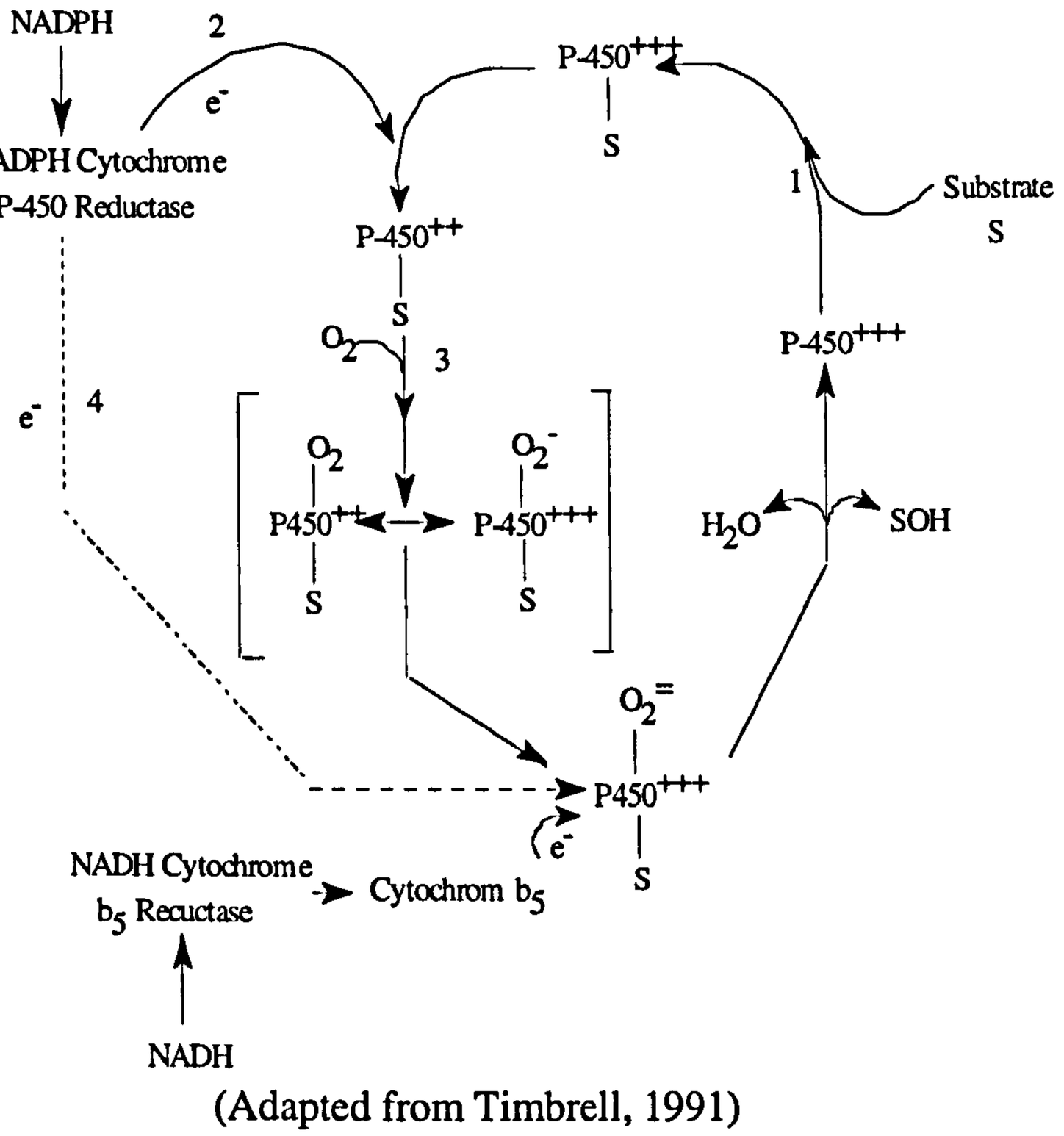
1.2.1 Phase I biotransformations

As mentioned previously phase I biotransformations can be divided into oxidative and reductive reactions which alter and create functional groups and hydrolytic reactions which cleave esters and amides to expose masked functional groups. The majority of the oxidative reactions involve the cytochrome P-450 dependent monooxygenases which are located in the endoplasmic reticulum. Other enzymes known to be important in these oxidation reactions are the flavin containing monooxygenases, alcohol dehydrogenase, aldehyde dehydrogenase, xanthine oxidase, monoamine oxidase and diamine oxidase.

Reductive and hydrolytic pathways are quantitatively less important than other pathways. The enzymes involve the cytochrome P-450 monooxygenases, nitroreductases, esterases and amidases.

A variety of phase I reactions are performed by cytochrome P-450, a super-family of haem-containing isozymes with distinct although overlapping substrate specificity (Gonzalez, 1992). The term cytochrome P-450 itself refers to a group of haem containing proteins which exhibit a major absorption band at 450 nm when reduced and complexed with carbon monoxide (Omura and Sato, 1964). The enzymes have been shown to be ubiquitous, present in all living organisms and in virtually all tissues. The enzyme is located particularly in the membranes of the smooth endoplasmic reticulum of the cell and is isolated in the so-called microsomal fraction which is formed from the endoplasmic reticulum when the cell is homogenised and fractionated by differential ultracentrifugation (Timbrell, 1991).

The overall reaction carried out by the cytochrome P-450 dependent monooxygenase system is shown below:



(Adapted from Timbrell, 1991)

All of the reactions require the presence of molecular oxygen and reduced nicotinamide adenine dinucleotide phosphate (NADPH) and involve four distinct steps:

1. addition of substrate to enzyme

2. donation of an electron

3. addition of oxygen and rearrangement

4. donation of a second electron and loss of water

The ability of the hepatic mixed function oxygenase system to metabolise xenobiotic substances can be altered to a remarkable degree by prior exposure of animals to a variety of different chemicals. Exposure to any of a large number of nonpolar compounds, which fall into two main groups typified by phenobarbital and 3-methylcholanthrene, leads to augmentation of the activity of the mixed function oxygenase system for specific types of substrates by a process termed "induction" (Remmer, 1972). In addition, a number of agents inhibit the hepatic mixed function oxygenase system by interfering with separate components. For example, SKF-525-A (β-diethylaminoethyldiphenyl propylacetate) and metyrapone block the system by competing for the substrate binding site on cytochrome P-450 (Buening and Franklin, 1976).

Cytochrome P-450 exists in multiple forms of monometric molecular weight approximately 45-55 kD (Gibson and Skett, 1994). The initial classification of P-450 isozymes was based on the small differences in the absorption maxima of the reduced carbon monoxide-complexed spectra which gave rise to P-450, P-448, P-456 etc. However, with the discovery of many more members of the P-450 family of enzymes, it became apparent that such a classification was inadequate. An understanding of the molecular biology of P-450s has been greatly aided by the tremendous progress in the 1970s and early 1980s in purifying many forms of P-450s and preparing highly specific antibodies against these enzymes. Unfortunately, this resulted in much confusion as to the identity of individual enzymes as no commonly agreed nomenclature existed. In fact, particular cytochrome P-450s would have several different names if isolated in different laboratories. However, cloning and sequencing technology developed rapidly, and it became possible to analyse cytochrome P-450 structure by isolation and sequencing of

cDNAs encoding multiple forms of the haemoprotein. These techniques allowed the development of a coherent nomenclature system which has now been universally accepted and uniquely identifies more than 200 different cytochrome P-450s. The basis of this unifying nomenclature system is divergent evolution and sequence similarity between the cytochrome P-450s into gene families and gene subfamilies. Families are designated by Arabic numerals, subfamilies by capital letters and individual P-450s are denoted by Arabic numerals. The different gene families differ from each other by > 60% amino acid similarity and > 60% similarity are placed in the same gene subfamily (Gonzalez, 1990; Nelson *et al.*, 1993). Some of the major P-450 isozymes and their characteristics in the rat is shown in Table 1.2. It should be noted that this nomenclature system does not depend on P-450 catalytic activities or function; thus each P-450 isozyme has the potential to catalyse more than one reaction whilst a single reaction can be catalysed by more than one isozyme of P-450.

1.2.2 Phase II biotransformations

After the introduction of a new functional group during phase I biotransformations the metabolite may undergo further metabolism via phase II pathways. However, these phase II biotransformations may also occur as the sole step in hepatic metabolism without an initial phase I reactions if a suitable functional group is already available on the parent compound (Tephly and Burchell, 1990). In the majority of cases phase II or conjugation reactions are detoxification processes. They involve the addition of small endogenous moieties such as sulphate, glucuronic acid or glutathione to the drug/metabolite which generally results in the formation of a more polar product for

excretion from the body. The reactions themselves are catalysed by a diverse group of enzymes and include glutathione transferases, sulphotransferases, glucuronyl transferases, acetyl transferases and catechol-O-methyl transferase. A common feature of many of these reactions is the requirement of some form of "activated" or energy rich intermediate e.g. uridine diphospho-glucuronic acid (UDPGA), 3'-phosphoadenosine-5'-phosphosulphate (PAPS), acetyl-CoA and S-adenosyl-methionine (SAM).

Table 1.2 Some P-450 Isozymes and Their Characteristics in the Rat

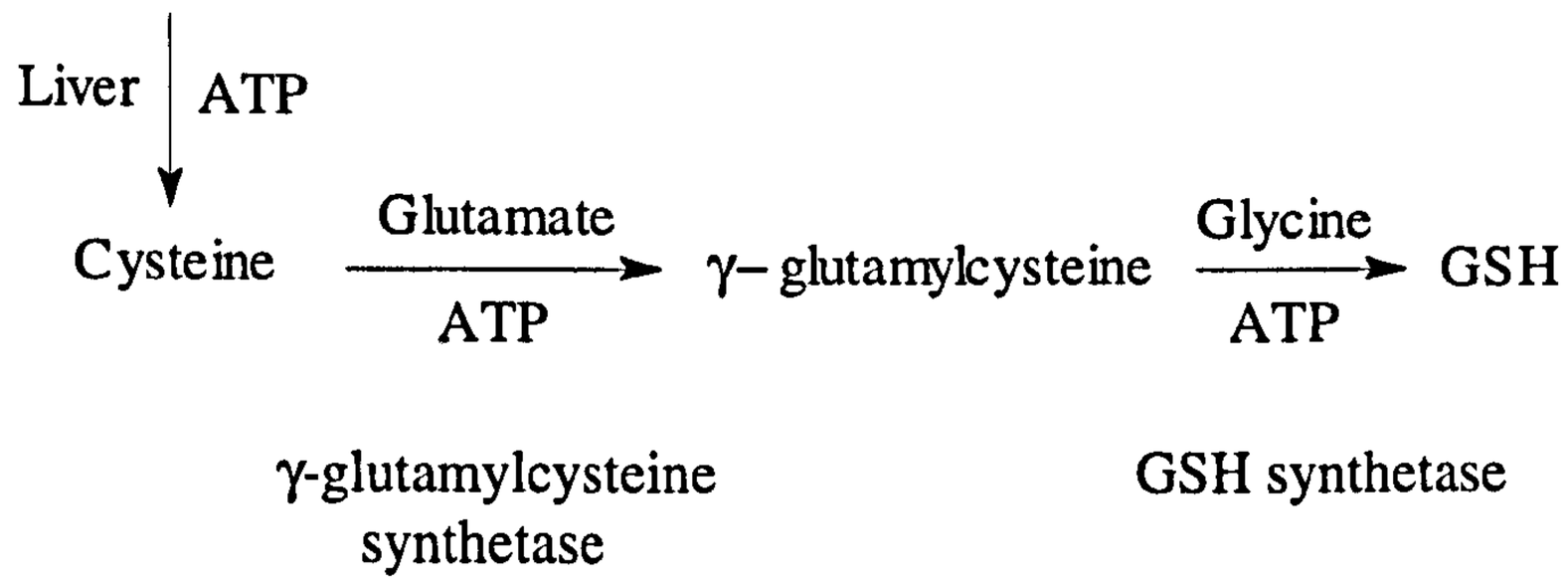
Family	Sub-family	Iso-enzyme	Trivial name	Liver induction	Sex specificity	Preferred reaction
1	A	1A1	c	3-MC	both	O-deethylation
		1A2	d	3-MC	both	6-hydroxylation
2	A	2A1	a	3-MC	female	N-demethylation
		2A2	-	3-MC	male	
	B	2B1	b	PB	both	N-demethylation
		2B2	e	PB	both	N-demethylation
		2B3	-	Et-OH	both	
	C	2C6	k	PB	both	N-demethylation
		2C7	f		both	N-demethylation
		2C11	h/16α		male	N-demethylation
		2C12	I/15β		female	15β-hydroxylation
		2C13	g		male	N-demethylation
	D	2D1	db1		both	4-hydroxylation
		2D2	db2			
		2D3	db3			
		2D4	db4			
	E	2E1	i	Et-OH	both	ρ-hydroxylation
	G	2G1	olf1			
3	A	3A1	pcn1	PCN	both	
		3A2	pcn2	PCN	male	
4	A	4A1	LAω1	Hypolipid-aemic drugs	both	ω-hydroxylation
		4A2	LAω2			
		4A3	LAω3			

(Extracted from Gonzalez, 1990). Abbreviations are: 3-MC, 3-methylcholanthrene; PB, phenobarbital; Et-OH, ethanol; PCN, pregenenolone 16α-carbonitrile.

In terms of drug induced toxicity, glutathione (GSH) conjugation is of particular interest. GSH is a tripeptide (γ-glutamylcysteinylglycine) and the most abundant non-protein thiol in almost all aerobic species, present in millimolar concentrations intracellularly. GSH reductase maintains more than 98% of intracellular glutathione in the reduced, thiol form (GSH) whilst the rest is present within the cell as mixed disulphides, as the oxidised, disulphide form (GSSG), or as thioethers. There are three key elements to the structure of GSH, which determine its metabolism and function. First, the N-terminal glutamyl and cysteinyl moieties are linked through the γ-carboxyl group of glutamate instead of the more common α-carboxyl peptide linkage. A second structural feature that impacts on metabolism is the presence of the C-terminal glycine, which protects the peptide against cleavage by intracellular γ-glutamylcylotransferase and completes the structural specificity of GSH-dependent enzymes. Finally, the most important portion of the tripeptide is the cysteinyl moiety. This provides the reactive thiol group, which is responsible for the many functions of GSH. These functions include: (1) detoxification of exogenous and endogenous compounds, such as reactive electrophiles and peroxides; (2) by reducing disulphide linkages of proteins and other molecular, GSH maintains the essential thiol status of these substances; (3) GSH serves as the major form in which cysteine is stored within the organism and provides a vehicle for the transfer of cysteine between organs and, (4) among its other functions are a role in leukotriene and prostaglandin metabolism and modulation of microtubule-related processes.

The synthesis of GSH from its three amino acid precursors, L-glutamate, L-cysteine, and glycine, requires 2 moles of ATP per mole of GSH. The synthesis takes place in the cytosol in two steps shown as follow:

Biosynthesis of glutathione

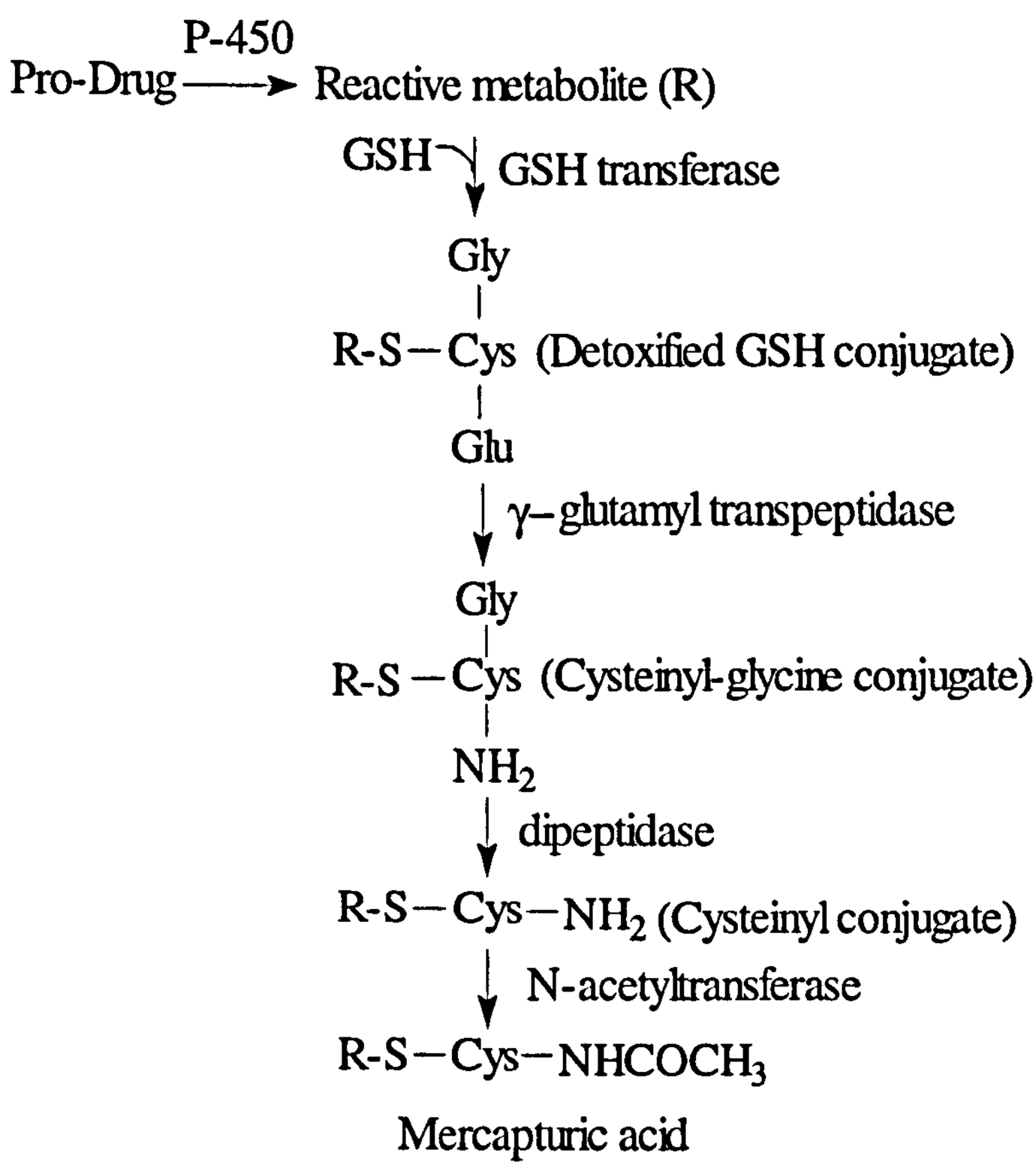


In the first step, which is rate-limiting, γ-glutamylcysteine synthetase catalyses the formation of an amide linkage between cysteine and the γ-carboxyl of glutamate. The reaction can be inhibited by nonallosteric competitive inhibition by the end-product, GSH, and can be limited by availability of cysteine, especially when GSH is consumed, leading to loss of feedback inhibition. In the next step GSH synthetase catalyses the reaction between glycine and the cysteine carboxyl of γ-glutamylcysteine.

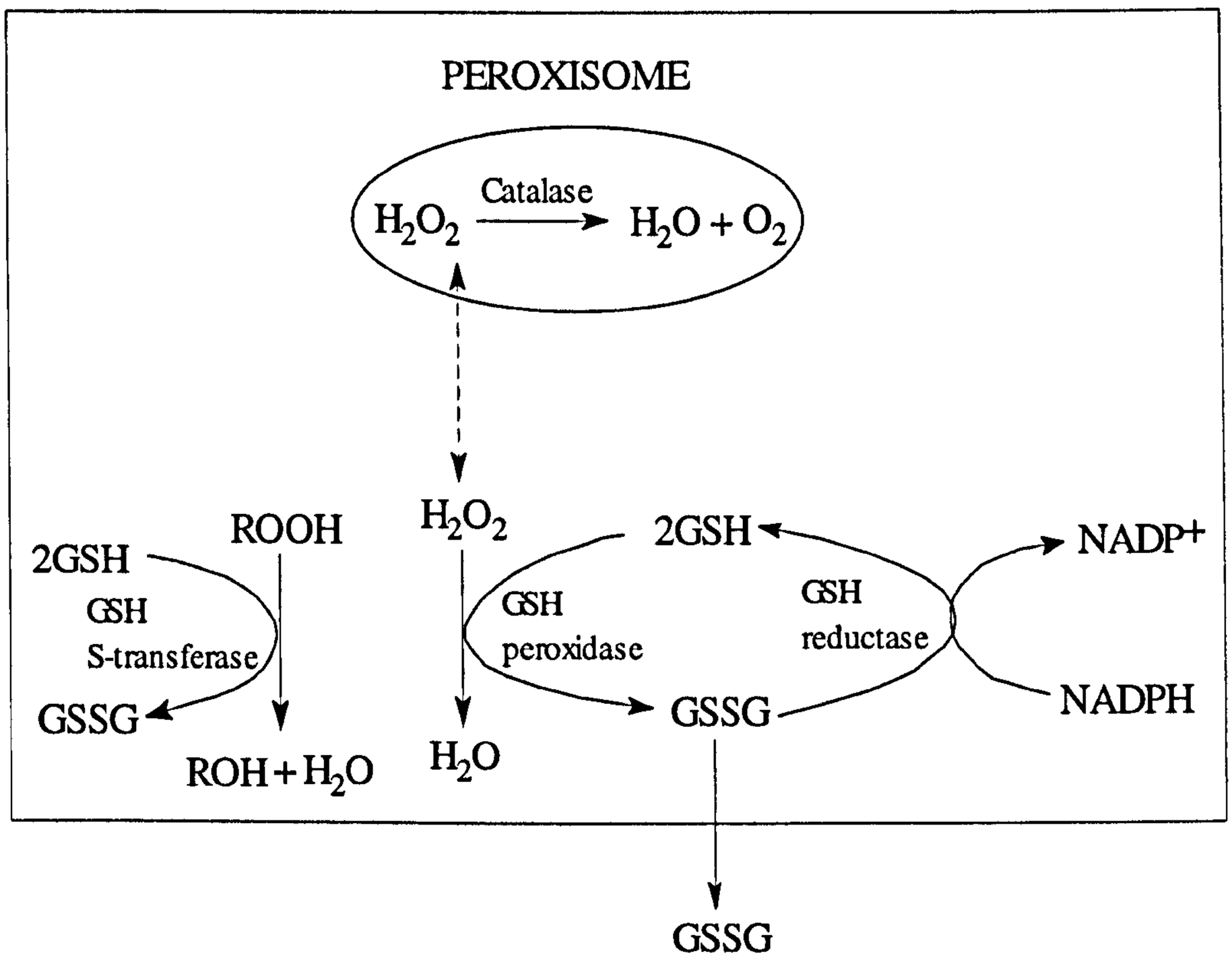
GSH is ubiquitous among mammalian cells, but is especially abundant in the liver where it reaches a concentration of 5 mM or more in mammals (Timbrell, 1991). The liver has the unique ability to utilise methionine as a precursor for cysteine, so that it can maintain GSH synthesis in the presence of cysteine deprivation. Approximately 10-15% of total intracellular GSH is located in the mitochondria, with 85-90% present in the

cytosol (Meredith and Reed, 1982). Depletion of mitochondrial GSH may be more likely to cause cell injury or death than cytosolic GSH depletion.

GSH is recognised as a protective device within the body for the removal of potentially toxic electrophilic compounds. Many cytotoxic xenobiotics and drugs are metabolised by phase I reactions to strong electrophiles such as epoxides, and these can react with GSH, either spontaneously or catalysed by GSH transferase to form (in general) non-toxic conjugates. The GSH conjugates can be further metabolised to yield a mercapturic acid and excreted into urine or bile as follow:



Oxidant stress is a physiological consequence of aerobic metabolism. The intermediates formed in aerobic organisms, such as superoxide and hydrogen peroxide, lead to the further production of reactive oxygen species that can oxidise membrane lipids and disrupt metabolic processes. As the cosubstrate for GSH peroxidase, GSH prevents peroxidation of membrane lipids. The reduction of endogenously produced hydrogen peroxide by GSH is catalysed by selenium-dependent GSH peroxidase and results in the formation of oxidised glutathione, GSSG as follow:



Hydrogen peroxide is reduced by GSH peroxidase in the cytosol and by catalase within the peroxisome. Organic peroxides are reduced by GSH peroxidase and by GSH transferases of the alpha class. The rapid reduction of GSSG to GSH is essential to maintain the thiol-disulphide redox state of the cell. With increasing oxidant stress, the

capacity of the GSH peroxidase/GSH reductase system may be overcome and intracellular accumulation of GSSG occurs. To maintain the redox status of the cell, GSSG is actively transported out of the cell.

Thus, GSH is a small molecule present in virtually all life forms with aerobic metabolism. The multiplicity of functions it fulfils would seem to reflect its presence within the cell early in evolution. The nucleophilic thiol group, imparts chemical versatility upon GSH such that it will react, probably as the thiolate ion, GS^-, with electrophiles. Thus, GSH protects cells by removing reactive metabolites.

1.2.3 Chemical cytotoxicity

As mentioned previously chemical toxicity may be due to the parent drug or metabolites formed as a result of biotransformation processes. If inadequately detoxified, these chemically reactive metabolites can combine with or damage cellular macromolecules such as proteins and nucleic acids resulting in various forms of toxicity, including cellular necrosis, carcinogenicity, teratogenicity and hypersensitivity (Park *et al.*, 1992). Cytotoxicity results when a compound produces direct irreversible damage to a cell. Cytotoxic reactions are usually dose-dependent, and therefore the level of toxicity is directly related to the amount of reactive compound present. As well as causing toxicity through covalent binding, direct cytotoxicity can be caused through redox cycling and the generation of reactive oxygen species, resulting in either lipid peroxidation and damage to the membrane integrity, or damage to important cellular enzymes (Hinson and Roberts, 1992).

Chemical-induced cytotoxicity has been linked to two major mechanisms that are commonly associated with cell damage/death. One is by irreversible binding of reactive metabolites to cellular macromolecules whilst the other involves lipid peroxidation as one of the most important organic expressions of oxidative stress induced by the reactivity of oxygen free radicals. Some workers consider that the lipid peroxidation damage is involved in most liver injury (Ohkawa *et al.*, 1979; Wolfgang *et al.*, 1990a, 1991). Thus, this second mechanism involves the mediation by reactive oxygen species such as superoxide anion, hydroxyl radical, and/or hydrogen peroxide which mediate peroxidation and oxidation of membrane lipids, nucleic acids and protein thiols. The subsequent injury to membrane structure can lead to irreversible cell damage and ultimately death. Toxicity may be a general effect of the oxidative cascade; however, lipid peroxidation is frequently stated as the mechanism. Multiple mechanisms have been described by which the reactive oxygen species may be formed. One mechanism which is frequently observed is a redox cycle. A metabolite is reduced to a species such as free radical and this reduction product is subsequently oxidised by molecular oxygen to reform the oxidised metabolite and superoxide anion. The latter product may be further reduced to hydrogen peroxide or hydrogen radicals.

Great efforts have been made to develop reliable analytical methods to determine the level of lipid peroxidation of a tissue homogenate, an organ or a whole organism. Many chemical and physical methods have been described to assess some of the chemical stages of the oxidative degradation of an unsaturated fatty acid when it is a component of a plasma membrane, subcellular fraction, or a circulatory macromolecule. These methods include: (1) measurement of malondialdehyde (MDA) production; (2)

evaluation of the UV absorption of conjugated dienes; (3) analysis of fluorescent products; (4) measurement of ethane and pentane formation; (5) detection of chemiluminiscence; (6) measurement of oxygen consumption, and, (7) measurement of the loss of polyunsaturated fatty acids from membrane phospholipids (Tappel, 1980; Jordan and Schenkman, 1982; Pryor and Castle, 1984; Dillard and Tappel, 1989). Although lipid peroxidation of biological samples may be assessed by many different chemical and physical methods, those based on the measurement of MDA formed from the breakdown of endoperoxides during the last stages of the oxidation of a polyunsaturated fatty acid, appear to be the most widely used. MDA in a free form or as a complex with various tissue constituents, can also be formed during the oxidative degradation by ionising radiation *in vivo*, and as a by-product of prostaglandin biosynthesis. However, peroxidation of unsaturated fatty acids is its major source. Direct spectrophotometry and high pressure liquid chromatography have been used to evaluate MDA formation. However, the reaction of MDA with thiobarbituric acid (TBA) to form a pink coloured adduct appears to be a more rapid, inexpensive and sensitive assay of MDA.

To study the mechanisms of toxicity mediated by chemicals we need to choose a suitable model system that allows the observation of toxic events and this is now discussed in the next section.

1.3 *In Vitro* Toxicity Testing and Organ Culture

1.3.1 *In vitro* toxicity testing

In recent years, tremendous progress has been made in our understanding of the biomolecular sciences. This has translated to multiple modifications and improvements in *in vivo* testing procedures which now give us tests that (a) are more reliable, reproducible, and predictive of potential hazards in humans, (b) use fewer animals, and (c) are considerably more humane than earlier test forms. At the same time, a multitude of *in vitro* test systems have been proposed, developed, and validated. Such systems hold great promise for the rapid, inexpensive evaluation of the potential toxicity of new and existing chemicals and pharmaceuticals. There are a number of considerations that are driving toxicology towards a broader use of *in vitro* test systems: (a) large numbers of new chemicals need to be screened for potential toxicity, (b) the rapidly increasing costs of laboratory animals and their upkeep, have resulted in spiralling costs for traditional *in vivo* models, (c) there is strong opposition from animal welfare and animal rights groups opposed to *in vivo* toxicity evaluation (Dagani, 1984; Push, 1984), (d) *in vitro* systems have the advantages of allowing for mechanistic studies into drug and xenobiotic toxicity and metabolism when the experimental conditions can be rigidly controlled (Grisham, 1979). The single most important advantage of *in vitro* tests is that they allow comparisons of the effects of cellular and organ exposure to drugs and chemicals to be extrapolated across species to include humans through the use of human cell culture from necropsy or biopsy material (Gad, 1994). In other words, such techniques have the

potential to allow the toxicological evaluation of compounds in animals and human on an equal basis, which cannot be achieved in classical *in vivo* toxicological testing.

There are a variety of *in vitro* test systems currently utilised for toxicity studies, each having a relatively specific set of applications. The major categories include microorganism, cell/tissue culture, and organ culture systems.

Microorganism systems employ various strains of cultured yeast (Zimmerman *et al.*, 1974) and bacteria (Ames, 1971; Legator and Malling, 1971) for the detection of mutational events. These screens are based on the hypothesis that carcinogen are mutagens and act because they modify DNA and lead to mutations, including neoplastic transformation. For example, point mutations have been effectively measured using unique mutant strains of S. typhimurium in the "Ames mutagenesis assay" (Ames *et al.*, 1975). In addition to recognising direct acting mutagens in this system, it is also possible to supplement the growth medium with a liver microsomal fraction (the "S-9" fraction of homogenised liver) along with other required co-factors necessary for mixed-function oxygenase activity and thereby generate reactive metabolites from promutagens (Ames *et al.*, 1975).

The use of cell/tissue culture techniques for toxicity testing was originally proposed by Pomerat and Leake (1954) who demonstrated that antihistamines were highly toxic to skin cultures, confirming the clinical observation of skin injury following topical application. Since then the applications of such techniques have been widespread and diverse. Unfortunately, however, many cell culture methods employ metabolically-

limited mammalian cells which have been subcultured repeatedly and lost many of the differentiated functions. For example, the microsomal enzymes such as P-450 enzymes, most often involved in biotransformation, are usually poorly expressed in such cells (Klingele, 1973; Paine, 1990; Wright and Paine, 1992).

Organ culture represents another *in vitro* technique which can be used for toxicity studies. Organ culture is defined as the maintenance or growth of tissue, organ primordia or the whole or parts of an organ *in vitro* in a way that may allow differentiation and preservation of the architecture and/or function (Federoff, 1967). This implies that the architecture which is characteristic of the tissue *in vivo* is retained, at least in part, in the culture (Freshney, 1983).

As the body's second largest organ (after the skin), the liver is undoubtedly one of the key organs in toxicology. The study of experimental hepatotoxicity has taken on great relevance in today's industrialised societies because of the rapidly growing rate of morbidity and mortality from liver disease due to drug and chemical exposures (Leevy *et al.*, 1980). A large variety of chemicals, including pharmaceuticals and environmental agents, are known to induce liver toxicity in the human population. For example, the widely used, over-the-counter analgesic paracetamol (acetaminophen), when taken in large quantities, is responsible for a significant number of hepatotoxic events often resulting in death whilst prolonged exposure to ethanol the key ingredient of alcoholic beverages leads to liver cirrhosis in both laboratory animals and man (Li, 1994).

1.3.2 *In vitro* liver models in toxicity testing

In vitro liver preparations are recognised as being very useful experimental models in pharmacotoxicological research; these *in vitro* preparations include the isolated perfused organ, isolated hepatocytes, subcellular fractions and tissue slices.

The isolated perfused liver represents the system closest to the *in vivo* situation whilst maintaining tissue architecture and cellular polarity. It makes it possible to study hepatic function without interference from the rest of the organism. The perfusate is recirculated and thus simulates blood circulation. Various parameters can be easily measured, e.g. pH, flow rate of the perfusate, oxygen consumption, bile excretion and enzyme release (Guillouzo and Guguen-Guillouzo, 1992). The perfused liver is a suitable tool with which to study the kinetics and metabolism of new drugs as well as drug interactions, provided that the drugs are rapidly cleared and metabolised. A major advantage of the perfused liver is that studies can be performed under carefully controlled conditions resembling those *in vivo*. However, this *in vitro* model is difficult to handle and its functional integrity is not maintained beyond a few hours (Guilouzo and Guguen-Guilouzo, 1992). Also, the neural and hormonal signals present *in vivo* are lost, as are the dynamic fluxes of nutrients provided from the diet and from peripheral tissues (Thurman *et al.*, 1988). In addition, it allows study of mouse/rat only due to the size considerations.

Isolated hepatocytes are extremely useful for a wide variety of biochemical, pharmacological and toxicological investigations which are described in a number of

reviews (Grisham *et al.*, 1978; Thurman and Kauffman, 1980; Rauckman and Padilla, 1987; McQeen, 1988). While studies with primary cell cultures of isolated hepatocytes have contributed to our knowledge of mechanisms of toxicity, they possess certain limitations. For example, an important problem with this technique is that the method of isolation most routinely utilised involves collagenase digestion which is not ideal for recovery of cell and enzymatic activities. This usually results in damaged cellular membranes, and disruption of transport systems. The lobular architecture and thus heterogeneous composition of the liver is lost, furthermore only parenchymal cells are used. The anatomical basis for the functional heterogeneity of the liver is destroyed when isolated hepatocytes are employed.

Tissue homogenates, microsomes and purified organelles can be prepared from whole livers or liver pieces. These models are useful to study mechanisms of hepatotoxicity. The liver 9000 g supernatant fraction (S9 fraction) fortified with cofactors is appropriate for expression of the cytochrome P-450-dependent microsomal monooxygenase system and has long been recognised as a good metabolising system for the activation of xenobiotics in *in vitro* mutagenicity testing procedures. Microsomes are widely used to identify drug-metabolic pathways, covalent binding and lipid peroxidation induced by hepatotoxins. The S9 fraction contains microsomes, cytosol and some mitochondria and therefore has the ability to perform the majority of phase I and II metabolic reactions. However, subcellular liver fractions are also functional for only a few hours (Guillozo and Guguen-Guillozo, 1992).

Therefore, a method for culturing liver under conditions where the architecture of the tissue is maintained and collagenase digestion is avoided, should provide significant further information regarding the mechanism(s) of toxicity of drugs and xenobiotics. Liver slices in organ culture can provide these advantages. In this system, the anatomical basis for the functional heterogeneity is preserved, since tissue organisation, i.e. cell-matrix and cell-cell interactions, is not disrupted. Thus, maintenance of hepatic architecture in liver slices makes it possible to study metabolism and toxicity in distinct regions of the liver lobule and to study interactions among different cell types absent in preparations of isolated hepatocytes. Furthermore, the slices can be prepared from a variety of species, including human, thus allowing the ability to estimate the potential for interactions in man. In addition, such techniques help to reduce the numbers of animals used. Overall, tissue slices offer a valid *in vitro* system for performing species comparisons and chemical-chemical interaction studies.

The recent development of the Krumdieck tissue slicer providing precision-cut tissue slices in a rapid and reproducible manner had led to new interest in the tissue slice system (Smith *et al.*, 1985). Precision-cut liver slices have been used in the study of a large number of hepatotoxins and results have concurred with previous *in vivo* and *in vitro* data (Azri *et al.*, 1990a). The classical hepatotoxins allyl alcohol and bromobenzene were used to demonstrate the applicability of liver slices to the assessment of *in vitro* cytotoxicity (Smith *et al.*, 1987). The toxicities of aflatoxin B_1 (Uwaifo, 1984), endotoxin (Richards and Saba, 1985), carbon tetrachloride (Azri *et al.*, 1990b), chloroform (Azri *et al.*, 1992), chlorobenzene (Fisher *et al.*, 1990), valproic acid (Fisher *et al.*, 1991a, 1994), cocaine (Connors *et al.*, 1990; Boelsterli and Goldlin, 1991),

paraquat (Togashi *et al.*, 1991), paracetamol (Miller *et al.*, 1993) and metronidazole (Sidelmann *et al.*, 1996) have been studied in liver slices. The metabolism of a number of drugs such as chlorobenzene (Barr *et al.*, 1991a and b), diazepam (Dale *et al.*, 1988), halothane (Ghantous *et al.*, 1990b; 1991b), caffeine, tolbutamide and phenytoin (Worboys *et al.*, 1997) have also been investigated in rat liver slices. Furthermore, xenobiotic metabolism and toxicity studies have also been conducted with human liver slices including dichlorobenzene (Fisher *et al.*, 1991b), theophylline (Salyers *et al.*, 1994), alcohol, coumarin and menadione (Price *et al.*, 1996). Thus, it is clear that precision-cut liver slices have an important role in the future of drug metabolism research.

1.3.3 Methods of organ culture

1.3.3.1 An overview of organ culture

Many factors influence organ culture and these include:

(1) Trauma

The object of organ culture is to maintain the structural and functional integrity of the explanted tissue. The careful manipulation of tissues is therefore an initial and important factor to minimise the possible loss of tissue organisation which may result from traumatic dissection or handling procedures.

(2) Size

The maximum size of the explants should be within the predicted the limiting radius of oxygen diffusion through a given tissue (Trowell, 1959).

(3) Age

The question of the influence of tissue age on the survival and differentiation of explants *in vitro* has been frequently raised in organ culture studies (Le Douarin, 1970).

(4) Temperature

Tissues are, in general, cultured at temperatures within the range of the body temperature of the animal from which they are derived, though tissues may survive and grow at other temperatures. The behaviour of tissues *in vitro* is optimal, therefore, within narrow temperature limits (Hodges, 1975).

(5) Gas phase

The composition of the gas phase is of considerable importance in the culture of tissues. In general, a gaseous environment of 5% carbon dioxide in air is used and is based on its being the usual concentration in the alveolar spaces of the lung (McLimans, 1972). Most culture media are formulated with a bicarbonate-buffer system and the bicarbonate concentration is adjusted such that a 5-8% carbon dioxide gas phase can be used to stabilise the pH of the medium.

(6) Media

The principle underlying the design of media for cell and organ culture systems is to provide an *in vitro* nutritional environment which approximates, as closely as possible,

to that occurring in the intact animal. The literature on media has been extensively reviewed and should be referred to for discussion of the basic requirements of *in vitro* cultures; namely, inorganic salts, trace elements, organic acid, amino acids, carbohydrates and vitamins (Waymouth, 1972, 1974).

(7) pH

The hydrogen ion content of most tissue culture media is based on the pH of the body fluid in the animals from which the tissue is derived and pH values optimal for mammalian and avian tissue have been found to lie within the range 6.8 to 7.8 (Waymouth, 1972). The majority of culture media are buffered with phosphates and carbon dioxide/bicarbonate or Hepes approximating to the normal physiological situation.

1.3.3.2 Culture of precision-cut liver slices

In order to improve the diffusion of oxygen and nutrients which is a critical factor in the survival of adult liver organ cultures, Freshney (1983) has underlined the advantages of a constant and gentle stirring of the medium and therefore recommended the use of roller cultures over static cultures. Smith *et al.* (1985) developed a dynamic organ culture system in which individual liver slices are supported on screens in rotating vials. This allows for the exposure of both sides of the slice to the gas phase and rotates the slice adhering to a stainless-steel mesh through the incubation media and atmosphere of the vial in a cyclical manner. This technique has been used successfully in studies of the toxic effects of volatile substances (Dale *et al.*, 1988; Ghantous *et al.*, 1990b,

1991b). Such a culture system, often referred to as roller culture, has been claimed to be superior to multiwell plate culture for maintaining precision-cut tissue slices (Fisher *et al.*, 1995a). This finding was not in line with the results reported by Olinga *et al.*, (1997) who found that the roller system was sensitive to bacterial infection and that some liver slices were floating in the glass vial and subsequently squeezed between the insert and the glass vial which may result in the decrease of viability. A drawback of this system is that there is no permanent contact of the slice with the incubation medium in which the test substance is dissolved. For metabolism studies, a system is preferred in which slices are continuously exposed to the compound under investigation; also the release of metabolites from slices into the medium should not be interrupted.

Another incubation system was described by Connors *et al* (1990) in which slices are placed individually, on stainless steel baskets in 24-well plastic tissue culture plates containing a teflon stir ball or ring on a gyratory shaker. Incubations were carried out in closed plastic chambers on individual aluminium heating blocks at 32 °C under constant oxygenation (95% O_2/5% CO_2). This incubation system caused mechanical damage and deformations on the surface of the slice. Dogterom (1993) modified this system such that slices were simply incubated individually in 24-well plates at a rotation speed of 90 rpm in a temperature controlled incubator (37 °C) under an atmosphere of 95% air /5% CO_2.

The optimal conditions for culturing slices with either nutrient-deprived or nutrient-enriched media, Hepes of bicarbonate-based media and whether different nutrient-enriched media were determined by Fisher *et al.* (1995a). They found that Waymouth's bicarbonate medium was one of the better media to use for either rat or

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mouse liver slices experiments. Scott *et al.* (1995) reported that Dulbecco's Modified Eagle's medium appeared to offer the most favourable conditions for metabolism in liver slices experiments.

1.4 Indicators of Viability and Toxicity in *In Vitro* System

In designing experiments for evaluating the viability of cultured tissue and its cytotoxic response to chemicals one is clearly faced with the problem of which indicators of viability to use. Commonly employed biochemical markers for a number of different hepatic lesions observed *in vivo* are listed in Table 1.3.

Table 1.3 Biochemical Indicators for Various Hepatic lesions

Lesion	Indicator change
Necrosis	Transaminase and dehydrogenase leakage into plasma
Steatosis	Fatty acid accumulation
Cholestasis	Alkaline phosphatase elevation in liver and plasma
Cirrhosis	Collagen production
Tumorigenesis	Increase or decrease in marker proteins/enzymes
Peroxisome proliferation	Palmitoyl-CoA oxidation (cyanide insensitive) and carnitine acetyltransferase induction

(Adapted from Tyson and Green, 1987)

Factors that influence the choice of indicator are as follows: (1) correspondence with the lesion *in vivo* being simulated, (2) the nature of the change anticipated (an early or late event, a reversible or irreversible change in structure or function), (3) the organelle(s) to be monitored, (4) sensitivity, (5) species specificity, (6) reproducibility and reliability, and (7) convenience.

The ideal test for cell viability is one which is easy and rapid to perform, which can be used in a wide variety of circumstances (different species and different nutritional states, etc.), which reflects a vital cell function, and which is sensitive to even minor disturbances of such function. While others have pointed out that there are no ideal viability parameters and no single test supplies full information about the metabolic competence of cultured liver cells (Krebs *et al.*, 1979), the criteria to be used should at least question the integrity and function of the plasma membrane and the metabolic performance of the cells.

1.4.1 Cell viability

The integrity of the plasma membrane is a key factor in cell function and viability. Tests of the integrity of the plasma membrane are the most widely used tests of cellular integrity, both for the initial isolation of tissue slices and in studies of xenobiotic-mediated toxicity. Indicators commonly used for monitoring the integrity of the plasma membrane in cytotoxicity studies are listed in Table 1.4.

Table 1.4 Indicators of Plasma Membrane Integrity

Indicator	Change	Permanency
Cell count	Fewer cells	Irreversible
Trypan blue	Cell stain	Irreversible
Cytosolic enzyme	Leakage to culture medium	Irreversible
K^+	Leakage to culture medium	Reversible

(Adapted from Tyson and Green, 1987)

1.4.2 Enzyme leakage

The ability of hepatocytes to retain intracellular enzymes is generally accepted as an indicator of irreversible damage to the plasma membrane. The measurement of enzyme activities is more precise than dye exclusion. Several enzymes from different subcellular locations are used for monitoring the loss of plasma membrane integrity. For example, the release of the cytosolic enzyme lactate dehydrogenase (LDH), the plasma membrane enzyme alkaline phosphodiesterase (PDE) and alanine aminotransferase (ALT), which is found in both the cytosol and the mitochondrial matrix, correlates well with the cell viability assessed by trypan blue exclusion for various cytotoxins (Story *et al.*, 1983). Leakage of LDH is probably the most common test of plasma membrane integrity performed in cytotoxicity studies. Its popularity is largely because it mimics the approach adopted in the clinical chemistry analysis of toxicity *in vivo*.

1.4.3 Leakage of ions

Increased permeability of cellular membranes to ions often precedes total loss of cell viability. Intracellular K^+ is a sensitive indicator of injury when compared with various other parameters of viability in freshly isolated cells (Baur *et al.*, 1975). The maintenance of the transmembrane Na^+ and K^+ ionic gradients is provided by the enzyme Na^+/K^+-ATPase, and it has been suggested that maintenance of these gradients may be sensitive indicators of plasma membrane integrity.

1.4.4 Energy conservation

Unless the cytotoxin acts externally on the plasma membrane without penetration into the cells, loss of membrane integrity is almost invariably preceded by subcellular changes. The detection and documentation of these changes is important and the status of energy conservation processes in the mitochondria is an overriding consideration (Krebs *et al.*, 1979). The indicator most commonly monitored in cytotoxicity studies with isolated hepatocytes that is dependent on the functional integrity of mitochondria is cellular ATP. Cellular ATP as an indicator of toxicity has been advocated by a number of authors (Smith *et al.*, 1988; Page *et al.*, 1992) whilst maintenance of ATP levels has been claimed to be a good prognostic indicator of success of hepatic transplantation (Sorrentino *et al.*, 1991). Measurement of O_2 consumption is also monitored but less frequently. Several processes and components which indicate functional integrity besides cellular ATP also depend intimately on the mitochondrial energy status and these are summarised in Table 1.5.

Table 1.5 Indicators Dependent on Energy Competence

Indicator	Compartmentation
ATP (or ATP/ADP)	Mitochondria, Cytosol
O_2 consumption	Mitochondria (predominantly) Endoplasmic reticulum
Pyridine nucleotide ratios	Mitochondria, Endoplasmic reticulum, Cytosol
Ureagenesis	Mitochondria, Cytosol
Gluconeogenesis	Mitochondria, Cytosol
Protein synthesis	Mitochondria, Endoplasmic reticulum
Lactate/pyruvate	Mitochondria, Cytosol

(Adapted from Tyson and Green, 1987)

1.4.5 Metabolic competence

Assessment of cell functional capabilities and metabolic competence often reveals subtle or early changes induced by a cytotoxin, which provide insight into reaction mechanisms that alter cell behaviour or proceed irreversible cell injury (Grisham, 1979; Krebs *et al.*, 1979; Krack *et al.*, 1985). Urea synthesis and protein synthesis are commonly used for this purpose. Protein synthesis has been proposed as a sensitive indicator of metabolic competence and is usually altered at earlier times or at lower concentrations of the cytotoxin than indicators of cell death (Gwynn *et al.*, 1979; Krack *et al.*, 1983, 1985).

1. 4.6 Nonspecific indicators

Nonspecific indicators of cytotoxicity are classified here as those that indicate a potential for injury without insight into the particular subcellular site(s). Reduced glutathione (GSH) levels, lipid peroxidation and covalent binding of the parent compounds or active metabolite to cell macromolecules are commonly measured indicators that fall into this category. GSH protects against cell damage by reacting with cytotoxins such as alkylating agents, oxygen-derived electrophiles and activated metabolites (see Section 1.2.2). A reduction in cellular GSH is a reflection of the potential for injury, not of cytotoxicity itself (Grisham, 1979).

With rat hepatocytes, loss of activity of P-450 enzymes, followed by loss of the mechanism for synthesising of P-450, appears to be one of the earliest changes to take place and might therefore be employed as a viability parameter (Bridges, 1981).

Morphological evaluation is a classic method to assess cytotoxic reactions, including cell death. Classic light microscopic detection of cell death utilises as its end-point irreversible changes in the nucleus (pyknosis, karyorrhexis and karyolysis) that are correlated with the catabolism of chromatin and the complete destruction of the nucleus. It is well established that blebbing of the plasma membrane may be an early step in the toxicity of certain agents to hepatocytes (Hayes and Pickering, 1985). Subcellular alterations can be demonstrated by electron microscopic examination including peroxisome and smooth endoplasmic reticulum proliferation, glycogen depletion and mitochondrion swelling (Guillouzo and Guguen-Guillouzo, 1992).

The hepatocyte couplet preparation offers a sensitive marker for the effects of toxins. The couplets are prepared by collagenase perfusion of rat liver selecting for hepatocytes that remain attached to one another, as initially described by Oshio and Phillips (1981). A unique feature of couplets, which represents the primary bile secretory unit, is the maintenance of structure and function, much as exists in the intact organ. The isolated rat hepatocyte couplet is particularly suited for studies using fluorescent microscopic or electrophysiological techniques (Boyer *et al.*, 1990).

1.5 Hepatotoxicity of Bromobenzene

A classical hepatotoxin, bromobenzene (BB), was chosen for evaluation in precision-cut rat liver slices system. It is a toxic industrial solvent which causes centrilobular hepatic necrosis in experimental animals. It may also cause renal damage and bronchiolar necrosis (Fakjian and Buckpitt, 1984; Monks and Lau, 1984).

The mechanism and toxicity of BB has been investigated for well over one hundred years. The urinary excretion of mercapturic acids was first reported in 1879 in animals treated with BB (Lau and Monks, 1988). BB has since proven to be a valuable tool to unravel the complexities involved in chemical-induced toxicities. For example, the importance of metabolic activation via the cytochrome(s) P-450, the role of GSH in the detoxification of reactive metabolites, and, the toxicological significance of covalent binding, enzyme inactivation and lipid peroxidation have all been illustrated in studies with BB. Thus, many of the principles involved in chemical-induced toxicity have been exemplified in studies with BB and many studies utilising BB have provided a wealth of

information which have advanced our knowledge and understanding of the mechanisms by which relatively inert chemicals cause toxicity.

The metabolism of BB is complex. BB is metabolised initially to an epoxide, 3,4-bromobenzene oxide, catalysed by cytochrome(s) P-450 (Figure 1.4). Epoxides are known to undergo rearrangement nonenzymatically to phenols, hydration by an epoxide hydrase to yield dihydrodiols, or conjugation with glutathione (GSH) catalysed by a glutathione S-transferase to ultimately yield mercapturic acids (Jollow *et al.*, 1974). It has been observed that radiolabelled bromobenzene became covalently bound to liver protein in the necrotic tissue of the centrilobular area.

It has been shown that BB-2,3- and 3,4-epoxides are formed as primary metabolites in cytochrome P-450 linked reactions, respectively. The responsible pathway for the hepatotoxicity is postulated to be that leading to 4-bromophenol via the 3,4-oxide (Thor *et al.*, 1980). Pretreatment of rats with the microsomal enzyme inducer phenobarbital results in increased hepatotoxicity of BB, increased urinary excretion of 4-bromophenol and greater covalent binding to liver protein (Jollow *et al.*, 1974). Treatment with phenobarbital also causes significant increases, both *in vivo* and *in vitro*, in the formation of glutathione conjugates formed directly from the 3,4-epoxide (Reid *et al.*, 1971; Lau and Monks, 1988). This suggests that the 2B type cytochrome P-450 is responsible for the primary oxidation of BB to reactive metabolites (Harauchi and Hirata, 1994). It was demonstrated that the 2,3- and 3,4-oxide bound to macromolecules at different rates with different proteins. Whereas bromobenzene-2,3-oxide readily and efficiently bound irreversibly to haemoglobin β-chain to a greater extent than the 3,4-

oxide, the latter bound more extensively to microsomal protein (Lau and Monks, 1988). This suggested that bromobenzene-2,3-oxide was more stable and could leave its site of microsomal synthesis and subsequently bind to soluble protein in other cells i.e., the β-chain of haemoglobin. In addition, the 2,3-oxide preferentially reacted with cysteine residues of the haemoglobin β-chain, whereas the 3,4-oxide reacted preferentially with histidine residues of the protein. This difference in the selective binding of the two epoxides may contribute to the observed differences in their hepatotoxicity (Lau and Monks, 1988). However, although the 2,3-oxide appears to be chemically more stable than the 3,4-oxide, the latter has been detected in the blood and in the incubation medium of hepatocytes *in vitro* after exposure to BB. This data means that the stability of the bromobenzene-3,4-oxide would allow it to leave the endoplasmic reticulum where it is formed and react with macromolecules in various parts of the cell.

One of the major urinary metabolites of BB is a mercapturic acid in which N-acetyl cysteine is conjugated on the 4-position (Figure 1.4). This results from an initial conjugation of the 3,4-oxide with GSH, followed by enzymatic removal of the glutamyl and glycinyl residues (see Section 1.2.2). Since early workers noted a correlation between the toxicity of various halobenzenes and their conversion to mercapturic acids, it seemed possible that a fundamental role of GSH might be to protect nucleophic sites in vital tissues from electrophilic attack by alkylating metabolites of foreign compounds. BB has been shown to produce a dose-dependent depletion of hepatic GSH both *in vivo* (Jollow *et al.*, 1974; Casini *et al.*, 1987; Maellaro *et al.*, 1990) and *in vitro* (Thor *et al.*, 1978; Jewell *et al.*, 1982; Menners *et al.*, 1991). These observations confirmed that GSH protects the hepatocyte against the reactive metabolite, bromobenzene-3,4-oxide. Once

the depletion of hepatic GSH attains the maximum due to conjugation with the chemically reactive 3,4-oxide, the remaining 3,4-oxide is free to bind covalently with the liver tissue macromolecules, leading to the development of liver injury. Studies *in vitro* revealed that the addition of GSH or a precursor of GSH (cysteine or N-acetyl cysteine) to the incubation mixture reduced the amount of covalent binding to protein, and the production of hydroxylated metabolites (Grewal *et al.*, 1996).

While there is no doubt that bromobenzene-3,4-oxide is the toxic metabolite of BB, recent studies have indicated that secondary quinone metabolites arise from the further oxidation of metabolites and these may be important in the toxic response (Slaughter and Hanzlik, 1991). Experiments carried out by Narashimhan *et al.* (1988) and Weller *et al.* (1988) demonstrated that much of the covalent binding of BB is due to the activity of quinone metabolites. However, covalent binding of these quinones probably does not contribute to the hepatotoxicity of BB (Lau *et al.*, 1984; Dankovic *et al.*, 1985), but quinones may contribute to the expression of early BB cytotoxicity by the induction of oxidative stress and related events (Casini *et al.*, 1987). It has been shown that bromoquinones and bromocatechols may be responsible for some of the covalent binding to protein and reaction with GSH (Buben *et al.*, 1988; Lau and Monks, 1988). Administration of primary phenolic metabolites does not cause hepatotoxicity. At least seven GSH conjugates have been identified as metabolites of BB and its primary phenolic metabolites.

BB toxicity in rat *in vivo* and in cultured hepatocytes involves the stimulation of lipid peroxidation, detected as an increase in malondialdehyde formation (Locke and

Brauer, 1991). The commonly used method is thiobarbituric acid assay of malondialdehyde production. It has been demonstrated that in primary cultures of hepatocytes BB induces a rapid depletion of GSH followed by the appearance of lipid peroxidation accompanied by liver cell death (Casini *et al.*, 1985; Wu *et al.*, 1995).

The mechanism of hepatotoxicity of BB therefore remains unclear. A number of mechanisms have been examined whereby the reactive intermediates of BB induce cytotoxicity. These include, among other mechanisms, arylation of critical macromolecules (Jollow *et al.*, 1974; Fisher *et al.*, 1993), lipid peroxidation (Casini *et al.*, 1985; Duthie *et al.*, 1994; Ferrandiz *et al.*, 1994), and deregulation of Ca^{2+} homeostasis (Casini *et al.*, 1987). It has been suggested that lipid peroxidation and not covalent binding could be a more likely cause for liver necrosis. Arylation of other low molecular weight nucleophiles such as coenzyme A and pyridine nucleotides also occurs and may be involved in the toxicity. BB is known to cause the inhibition or inactivation of enzymes containing SH groups. It also causes increased breakdown of phospholipids and inhibits enzymes involved in phospholipid synthesis. Arylation of crucial sites on the plasma membrane may also occur. This indicates that the toxicity of BB is complex and involves many events.

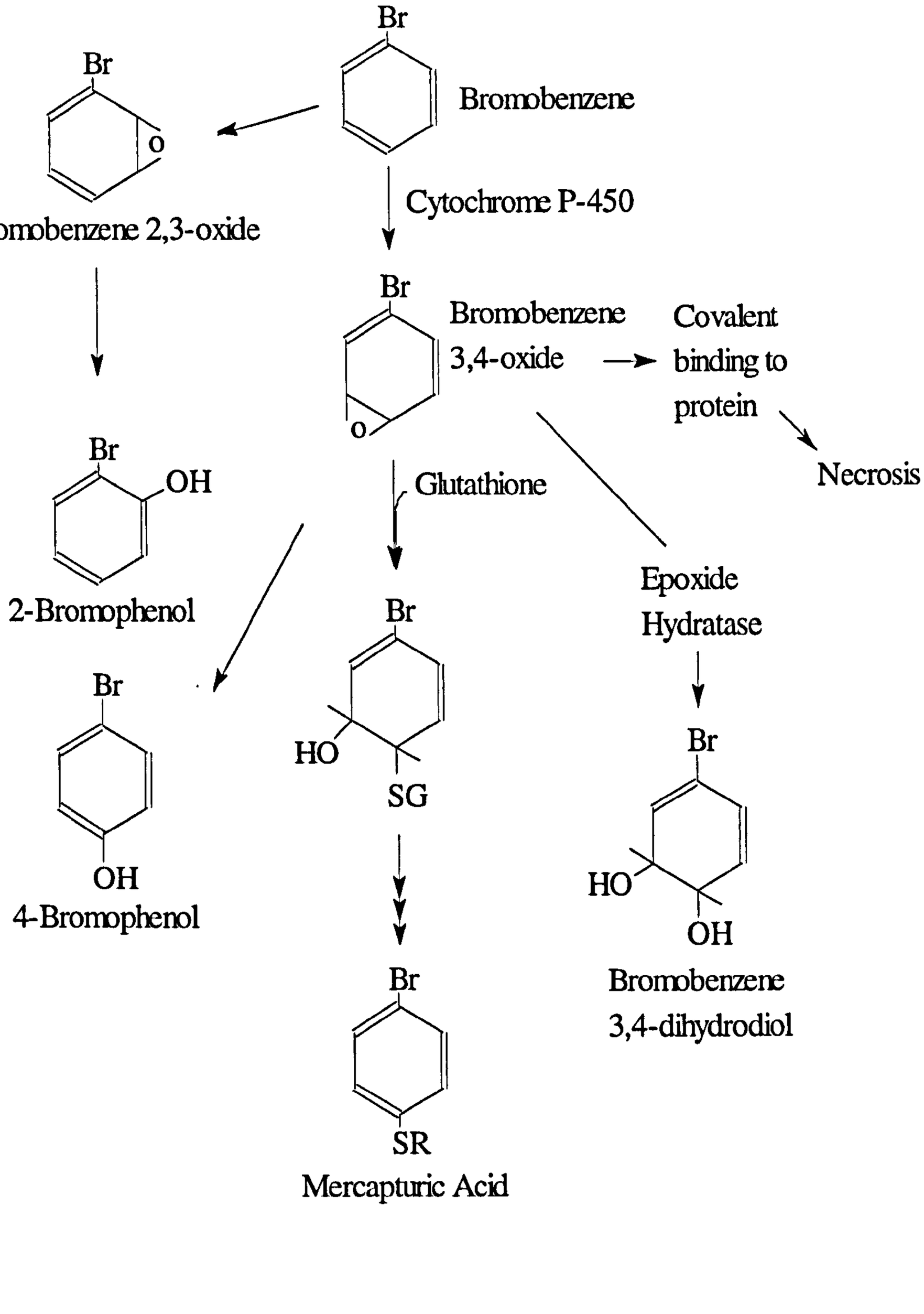


Figure 1.4 Metabolism of bromobenzene. The bromobenzene 2,3-oxide and 3,4-oxide may undergo chemical rearrangement to the 2- and 4-bromophenol, respectively. Bromobenzene 3,4-oxide may also be conjugated with glutathione and in its absence react with tissue protein. An alternative, detoxification pathway is hydration to the 3,4-dihydrodiol via epoxide hydrolase (From Timbrell, 1991).

1.6 Garlic and Its Biological Actions

Garlic is a member of the lily family and its botanical name is *Allium sativum*. Over the centuries, garlic has acquired a reputation in the folklore of many cultures as a formidable prophylactic and therapeutic medical agent. In ancient civilisations, including the Egyptian, Greek, Roman and Chinese, garlic was used for heart problems, headache, bites, worms, wounds, ulcers, and tumours (Sumiyoshi and Wargovich, 1989; Dausch and Nixon, 1990). Garlic is one of the most researched medicinal plants such that over the past 100 years, more than 1300 research articles have been published (Agarwal, 1996). Approximately 75% of these have been published since 1980, and have focused primarily on the cardioprotective, anticancer, antibacterial activity and antimicrobial properties of garlic. Of course the agents responsible for the these activities will vary in type and concentration depending upon the preparation, processing and source of the garlic.

1.6.1 Organosulphur compounds in garlic

The quality of garlic or garlic preparations is believed to be conveyed by certain substances which are responsible for its effects on health and its flavouring characteristics. Garlic contains high levels of phosphorus, potassium, sulphur, and zinc, moderate levels of selenium and vitamins A and C, and, low levels of calcium, magnesium, sodium, iron, manganese and B-complex vitamins. In addition, many compounds have been identified and isolated from garlic extracts including 33 sulphur compounds and 17 amino acids (Agarwal, 1996). Although whole garlic bulbs contain

several primary constituents regarded as precursors of beneficial substances (e.g., alliin and γ-glutamylpeptides), constituents in processed garlic may be even more complex. This is due to enzymatic and chemical cleavage, as well as to rearrangements and degradations that arise when different procedures are used for the preparation of commercial products. Furthermore, products and ingredients may change during prolonged storage, with volatile substances partially or even completely lost. All of the substances of interest are sulphur-containing compounds (Table 1.6).

Garlic is known to be one of the richest sources of sulphur compounds among all vegetables (Dausch and Nixon, 1990). γ-Glutamylcysteines, the parent compounds of alliin, occur abundantly in garlic cloves, apparently as a reserve pool that can increase its levels during storage and sprouting (Koch and Lawson, 1996). These compounds have been found to be fairly stable when cloves are maintained at room temperature, but when cloves are stored at 4-6 °C, they can be rapidly oxidised to alliin and isoalliin. Garlic contains alliin (S-allyl cysteine sulphoxide) and an enzyme, alliinase, which catalyses the formation of allicin (Figure 1.5). Allicin is a very unstable compound (Sumiyoshi and Wargovich, 1989) and decomposition proceeds along several pathways depending on the way garlic is prepared. Allicin is a very reactive compound and it undergoes a variety of spontaneous reactions in different solvents or media principally to diallyl trisulphide and diallyl disulphide (Figure 1.6). Allicin is also a strong oxidising agent and reacts rapidly with cysteine to form S-allyl mercaptocysteine and is rapidly converted into allyl mercaptan upon incubation with human whole blood (Lawson *et al.*, 1992). Ajoene (4,5,9-trithidodeca-1,6,11-triene 9-oxide) was originally discovered upon incubation of chopped garlic in methanol as a new compound which had remarkable activity toward

inhibition of blood platelet aggregation (Apitz-Castro *et al.*, 1983) and is formed from allicin. The most dramatic change that occurred during long periods of preparation of aged garlic extract (AGE) was the complete and quantitative hydrolysis of γ-glutamyl-S-allylcysteine (initially present in the garlic cloves) to S-allyl cysteine (SAC) in about 90 days (Koch and Lawson, 1996). The SAC formed was stable throughout the remainder of the 2 years. Garlic extracts and oils also contain a number of other organosulphur compounds including sulphide, disulphides and trisulphide, essentially breakdown products of allicin and alliin. These compounds have been studied to determine their role in various metabolic pathways. The uptake of diallyl disulphide by the liver is rapid when injected into mice, with peak uptake occurring at 90 min (Pushpendran *et al.*, 1982). In addition, over 80% of radiolabelled diallyl disulphide was converted to sulphates, presumably by hepatic mixed function oxidases.

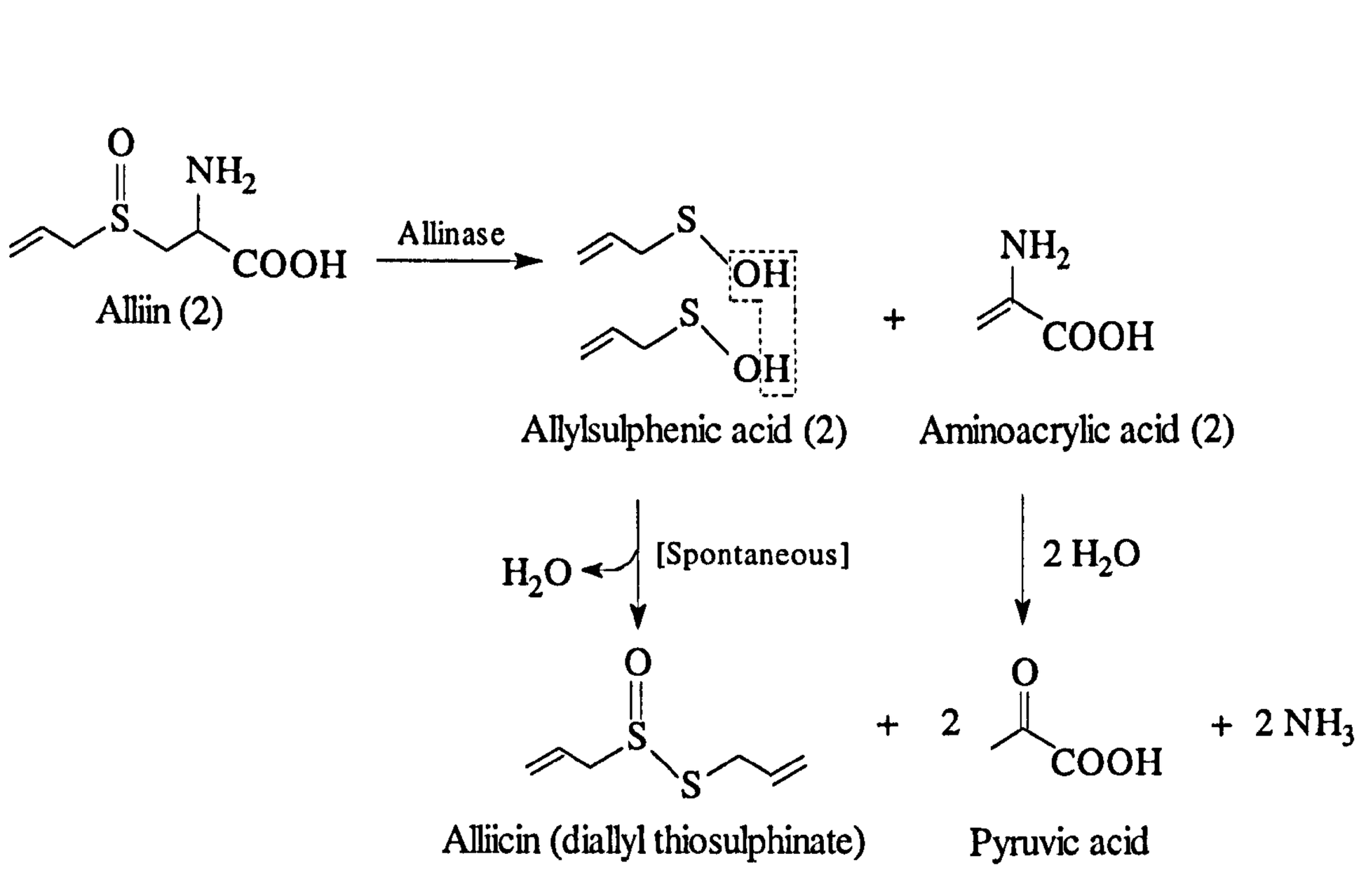
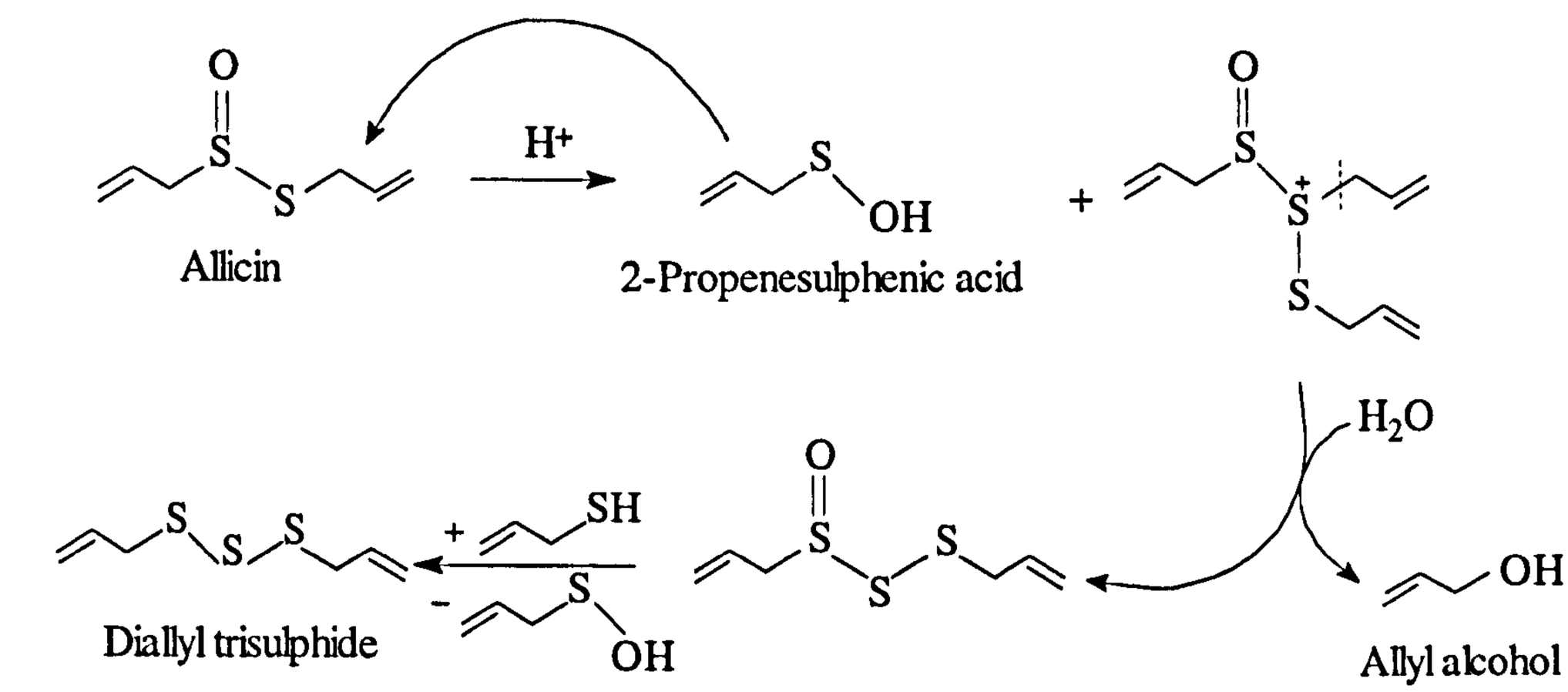


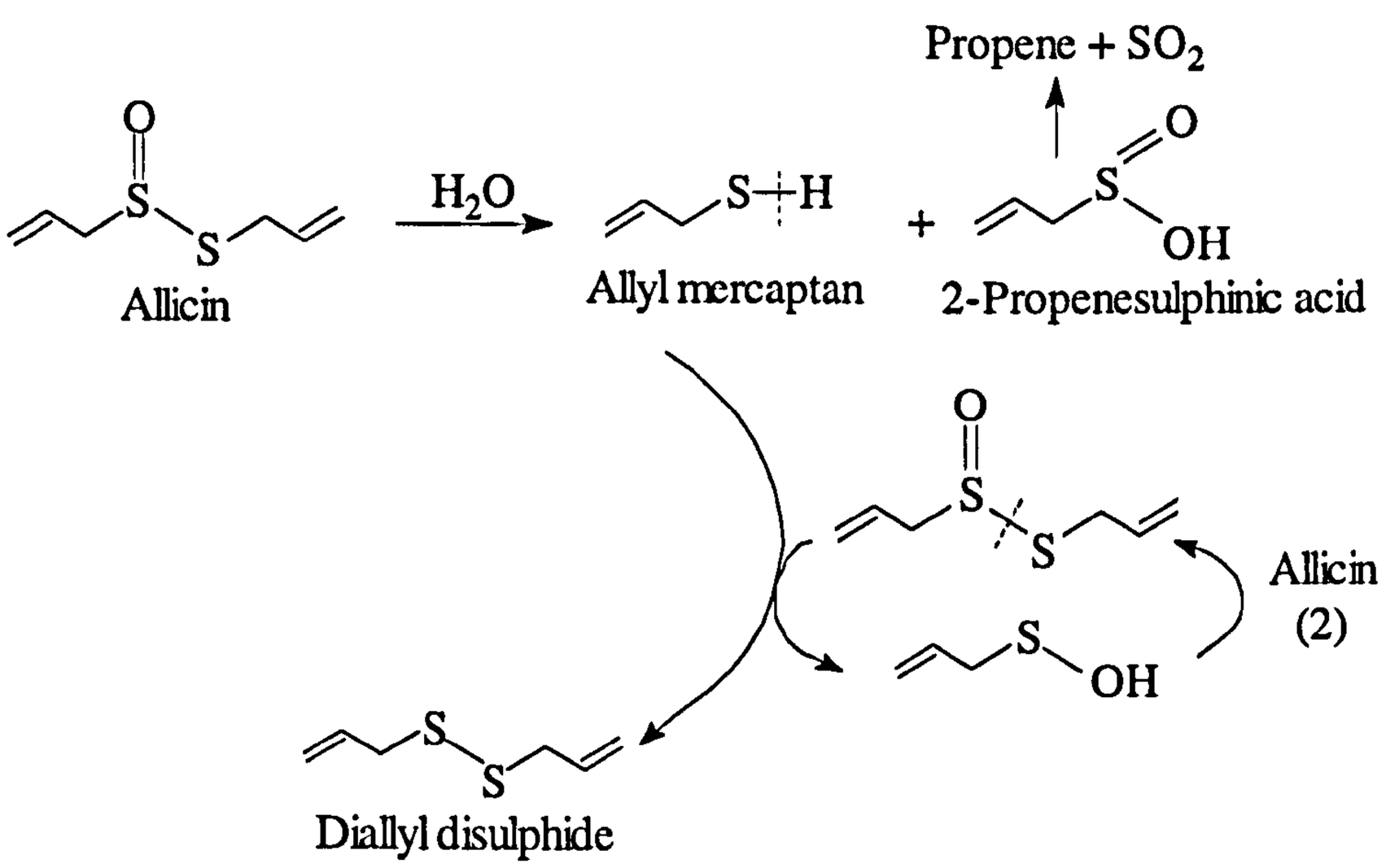
Figure 1.5 Formation of allicin from alliin. (From Koch and Lawson, 1996)

A



Overall: 3 allicin + H_2O $\xrightarrow{[-2\ O]}$ 2 diallyl trisulphide + 2 allyl alcohol

B



Overall: 3 allicin + H_2O $\longrightarrow$ 2 diallyl disulphide + 2 propene + 2 SO_2

Figure 1.6 Mechanisms for the spontaneous transformation of allicin to diallyl trisulphide (A) and diallyl disulphide (B). (From Koch and Lawson, 1996)

Table 1.6 Some Major Sulphur Compounds in Garlic

Name	Structural formulae
Alliin (S-allylcysteine sulphoxide)	CH_2=CH-CH_2-S(O)CH_2-CH(NH_2)CO_2H
Allicin (diallyl disulphide-oxide)	CH_2=CH-CH_2-S(O)-S-CH_2-CH=CH_2
Ajoene	CH_2=CH-CH_2-S(O)-CH_2CH=CHS-SCH_2CH=CH_2
Allyl propyldisulphide	CH_2=CH-CH_2-S-S-CH_2-CH=CH_2
Dipropyldisulphide	CH_3-CH_2-CH_2-S-S-CH_2-CH_2-CH_3
Diallylsulphide (DAS)	CH_2=CH-CH_2-S-CH_2-CH=CH_2
Diallyldisulphide (DADS)	CH_2=CH-CH_2-S-S-CH_2-CH=CH_2
Dimethyldisulphide	CH_3-S-S-CH_3
Dimethyltrisulphide	CH_3-S-S-S-CH_3
Allylmercaptan	CH_2=CH-CH_2-SH
S-Allyl cysteine (SAC)	CH_2=CH-CH_2-S-CH_2-CH(NH_2)CO_2H
S-Allylmercaptocysteine	CH_2=CH-CH_2-S-S-CH_2-CH(NH_2)CO_2H
S-Propyl cysteine	CH_3-CH_2-CH_2-S-CH_2-CH(NH_2)CO_2H
S-Ethyl cysteine	CH_3-CH_2 -S-CH_2-CH(NH_2)CO_2H

1.6.2 Garlic preparations

The benefits it is believed to convey have led to the marketing of numerous commercial garlic preparations over the last decades. Fresh garlic can offer the same advantage as commercial preparations but disadvantages include sprouting or rotting with prolonged storage, variable quality depending on different cultivation areas and

climate conditions, stomach complaints after consumption, and an unpleasant odour. Garlic preparations are manufactured by the pharmaceutical and natural products industries in various forms and are widely available in pharmacies and health food stores. The most important garlic preparations made from raw garlic are:

pressed juice (raw garlic juice, heated garlic juice)

garlic oil

dry powder (powder, granules, pellets)

oil macerates (oily solution)

pickled garlic (garlic cloves in vinegar/salt)

crushed or chopped garlic

aged alcoholic garlic extract (liquid or powder)

Depending on the type of processed garlic, different substances may serve as the main identifying compounds of the preparations. In order to deliver the potential advantages of garlic preparations over fresh garlic, they should contain sufficient and standardised amounts of biologically active compounds. The main problems in this regard arise from the fact that correlations between specific ingredients and their beneficial effects have not been clearly identified. In fresh garlic, the main "prodrug" principles are believed to be the alliin/alliinase and the cysteine-containing γ-glutamylpeptide systems, which are substantially preserved only in dry powder preparations, whereas in other garlic products tertiary alliin-derived sulphur-containing compounds would be the essential active ingredients.

A problem concerned with the unpleasant odour of garlic is directly related to its medicinal value (Koch, 1989). Many manufactures of garlic preparations claim that their product is "odourless" or "odour minimised" because they do not cause odour problems when consumed. This perhaps may be true of preparations made with "aged garlic". Aged garlic extract (AGE) is prepared by storing sliced garlic in 15-20% aqueous ethanol for 18-20 months at ambient temperature, followed by filtration and concentration to dryness at low temperature (Hirao *et al.*, 1987; Nakagawa *et al.*, 1989; Amagase and Milner, 1993) and sold in both dry (tablets and powder capsules) and liquid forms. The final liquid extract has been reported to contain 10% (w/v) ethanol and small amounts of several water-soluble sulphur compounds: S-allyl cysteine (0.62 mg/g), S-1-propenyl cysteine (0.15 mg/g), S-methyl cysteine (0.11 mg/g), γ-glutamyl-S-allylcysteine (0.28 mg/g), γ-glutamyl-S-1-propenylcysteine (0.17 mg/g), S-allylmercaptocysteine (0.04 mg/g), cysteine (0.01 mg/g), and less than 0.02 mg/g of alliin (Hansen *et al.*, 1993; Lawson, 1993). Others reported different data as following: alliin (1.7 mg/g), allicin (< 0.01 mg/g), ajoene (< 0.01 mg/g), and S-allyl cysteine (2.1 mg/g) (Imai *et al.*, 1994). The AGE liquid that used in this study contained SAC at 1.47 mg/ml. The key substance in AGE, however, has been proposed to be S-allylcysteine, a stable and naturally occurring water-soluble organosulphur compound generated by hydrolysis of γ-glutamyl-S-allyl cysteine (Reuter *et al.*, 1996).

1.6.3 Therapeutic effects of garlic

Garlic juice and oil, organic or aqueous extracts, and various constituents have all been reported to possess medicinal properties. Most studies on garlic have been primarily

in the fields of cardiovascular and cancer research. Evidence suggests that garlic and its compounds play a potential role in several areas.

Of all the effects of garlic and its compounds that have been reported over the years, perhaps the most widely reported are on cardiovascular disease. According to recent studies, garlic by appropriate application may decrease blood lipid concentrations (Abuirmeileh *et al.,* 1991; Yeh and Yeh, 1994; Gebhardt, 1995; Neil *et al.,* 1996; Steiner *et al.,* 1996), suppress platelet aggregation (Apitz *et al.,* 1983; Abuimeileh *et al.,* 1991; Lawson *et al.,* 1992; Morris *et al.,* 1995), protect against LDL oxidation induced by free radicals (Horie *et al.,* 1992; Imai *et al.,* 1994; Kojima *et al.,* 1994; Yamasaki *et al.,* 1994; Grune *et al.,* 1996; Ide *et al.,* 1996; Prasad *et al.,* 1996; Geng and Lau, 1997), increase capillary blood flow (Wolf and Reim, 1990) and lower elevated blood pressure levels (McMahon and Vargas, 1993; Ogawa *et al.,* 1993). This means that development of arteriosclerosis can be prevented or an already existing condition favourably influenced.

In recent years, garlic preparations have been found to protect against the occurrence of certain cancers (Sparnins *et al.,* 1988; Welch *et al.,* 1992; Li *et al.,* 1995; Ishikawa *et al.,* 1996; Sundaram and Milner, 1996; Polasa and Krishnaswamy, 1997; Schaffer *et al.,* 1996, 1997; Sigounas *et al.,* 1997). Growth inhibiting effects of garlic on bacteria, fungi, protozoa and viruses have also been shown *in vivo* and *in vitro* (Lawson and Wang, 1993; Shoji *et al.,* 1993; Urbina *et al.,* 1993; Davis *et al.,* 1994). In addition, evidence has been presented to suggest that garlic preparations have significant enhancing effects on the immune system (Brosche and Platt, 1993, 1994), antiinflammatory effects (Chung, 1985), antihypoglycemic effects (Kiesewetter and Jung,

1992), antiageing effects (Moriguchi *et al.*, 1996, 1997) and radioprotective effects (Singh *et al.*, 1995).

Garlic preparations and related organosulphur compounds have also been found to protect against certain cytotoxicities. For example, a garlic extract has been shown to inhibit the cardiotoxicity of doxorubicin in mice as a result of its antioxidant properties (Kojima *et al.*, 1994). Nakagawa *et al.* (1989) reported that AGE and SAC within garlic protect liver damage induced by paracetamol and carbon tetrachloride in mice. SAC has also been reported to protect rat hepatocytes from injury caused by carbon tetrachloride in culture (Hikino *et al.*, 1986) and to protect against paracetamol-induced toxicity in mice (Wang *et al.*, 1996). Also, fresh garlic (Wang *et al.*, 1996) and diallylsulphide (Hu *et al.*, 1996a; Lin *et al.*, 1996) protect against paracetamol hepatotoxicity in mice.

The mechanisms by which the active principles of garlic produce these effects are not clear. However, the active principles present in garlic have been identified to be mainly sulphur-containing compounds which are highly reactive with cellular-SH groups. Garlic's ability to affect sulphur metabolism may be indicative of a function in detoxification pathways. The conjugation of toxic chemicals with glutathione and cysteine S-conjugates represents a major function of cellular defence. Several glutathione-dependent bioactivation mechanisms are now recognised. As a result, garlic and its compounds have been found to modulate phase I and phase II biotransformation enzymes, some enzymes are inhibited while others are stimulated. A number of investigators have reported that garlic and its relative constituents inhibit the activities of some cytochrome P-450 isozymes and glutathione reductase (Brady *et al.*, 1991; Dalvi,

1992; Kwak *et al.,* 1995; Reicks and Crankshaw, 1996; Jin and Baillie, 1997; Guyonnet *et al.,* 1999). For example, it is well known that the toxicity of paracetamol results from its bioactivation to the toxic metabolite N-acetyl-p-benzoquinone imine by cytochromes P-450. The protective effect of garlic may be due, at least in part, to inhibition of this bioactivation since some garlic constituents inhibit some isoforms of cytochrome P-450 (Lin *et al.,* 1996; Wang *et al.,* 1996). On the other hand, the activities of glutathione S-transferase, epoxide hydrolase and glutathione peroxidase are stimulated by garlic preparations (Haber *et al.,* 1994; Dragnev *et al.,* 1995; Singh and Rao, 1995; Hatono *et al.,* 1996; Kim *et al.,* 1996; Hu and Singh, 1997).

1.7 Aims of These Studies

The aims of these studies were as follows:

(1) In the light of the above discussion it is evident that *in vitro* systems employing cultured liver slices offer huge potential for application to hepatotoxicity studies. Such a system, in which a higher level of biological complexity exists, may also facilitate extrapolation to the *in vivo* level. Thus, one of the objectives of this study was to develop and validate an *in vitro* organ culture system of adult rat liver for hepatotoxicity studies. In this regard, the Krumdieck tissue slicer and a simple shaking incubation system were used to overcome many problems associated with earlier organ culture methods of adult rat liver.

(2) When a model for organ culture of adult rat liver was established, validation of this system for *in vitro* hepatotoxicity studies was attempted. The classical hepatotoxin bromobenzene was chosen as a model.

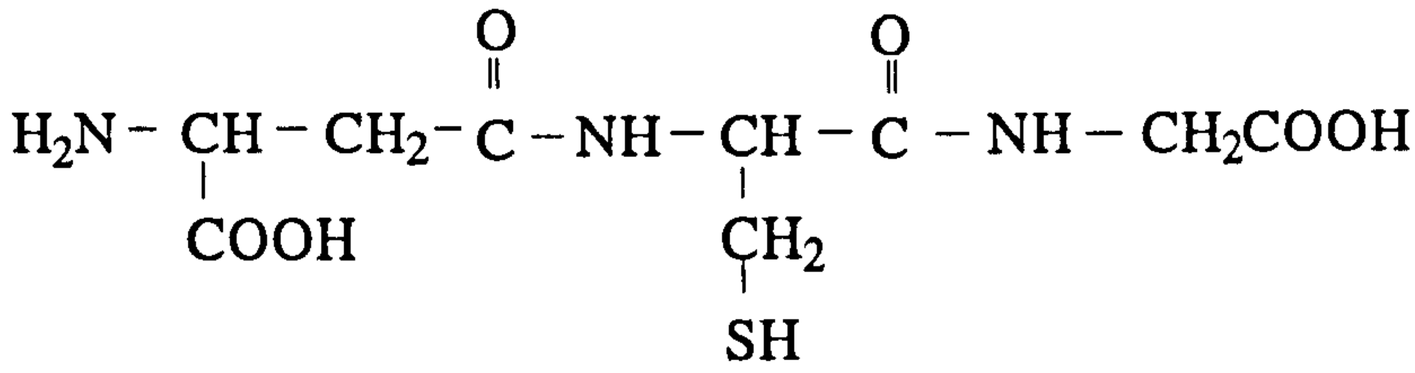
(3) After establishing and validating the system of cultured rat liver slices for measuring hepatotoxicity using the known hepatotoxin bromobenzene, the effects of AGE and its major organosulphur constituent SAC on BB-induced liver damage were assessed. AGE or SAC were added to liver slices *in vitro*, or AGE was fed to rats for 7 days prior to the preparation of slices.

(4) N-acetyl cysteine (NAC) was introduced as a mucolytic agent in the 1960s and has since found wide application as a cytoprotective agent in acute paracetamol poisoning. The metabolic pathway of BB toxicity is similar to that of paracetamol in that it involves bioactivation by cytochrome P-450 and conjugation of electrophilic metabolites with GSH to yield mercapturic acids. Also, the chemical structure of SAC is similar to that of NAC (Figure 1.7). Thus, the ability of NAC to protect against BB-induced toxicity was also examined. It has been suggested that NAC reduces the severity of liver necrosis due to its direct or indirect (via cysteine or GSH) nucleophilic capacity to conjugate and detoxify reactive metabolites (Bruno *et al.*, 1988; Simko *et al.*, 1992; Dai and Cederbaum, 1995).

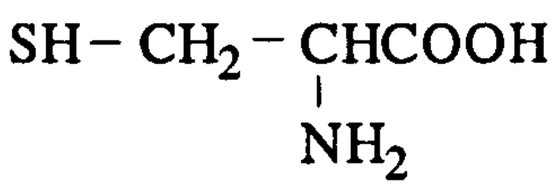
(5) As discussed in Section 1.5, GSH plays an important role in protection against hepatotoxicity induced by BB. GSH conjugates with the toxic metabolites of BB and BB toxicity only occurs after cellular stores of GSH have been deleted. A mechanism for

the antihepatotoxic activity of AGE, SAC and NAC could be their ability to restore hepatic GSH concentrations. Thus, the effects of AGE, SAC and NAC on BB toxicity in GSH-depleted hepatocytes were further investigated using buthionine sulphoximine (BSO). BSO is a potent and specific inhibitor of γ-glutamylcysteine synthetase and thus inhibits GSH biosynthesis and causes irreversible depletion of cellular GSH content (Griffith, 1982; Hammond and Fry, 1996). Furthermore, BSO has been used extensively as a specific depletor of tissue GSH content in studying the mechanisms of xenobiotic-induced toxicity.

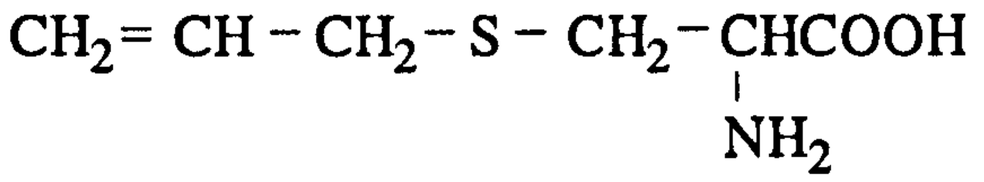
GSH (γ-glutamylcysteinylglycine)



Cysteine



SAC



NAC

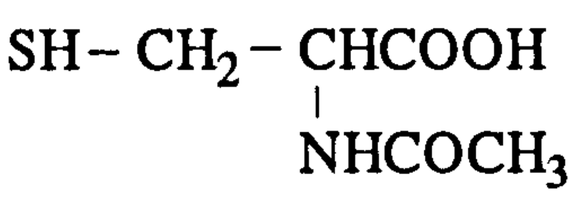


Figure 1.7 Structural formulae of glutathione (GSH), cysteine, S-allyl cysteine (SAC) and N-acetyl-L-cysteine (NAC).

CHAPTER 2

MATERIALS AND GENERAL METHODS

2.1 Chemicals

Aged Garlic Extract (AGE, Kyolic®) and S-allyl cysteine (SAC) were generously provided by Wakunaga of America Co. (Mission Viejo, CA). AGE was formulated by soaking sliced raw garlic (*Allium sativum*) in aqueous ethanol for more than 10 months at room temperature. The content of water-soluble compounds (expressed as dry weight) is as follows: alliin (1.7 mg/g); allicin (< 0.01 mg/g); ajoene (< 0.01 mg/g), and, S-allyl cysteine (1.47 mg/g). Oil-soluble compounds such as diallyl sulphide are present in AGE at only very low concentrations.

N-acetyl-L-cysteine (NAC), adenosine 5′-triphosphate (ATP), ammonium persulphate, bovine serum albumin (BSA, fraction V), bromobenzene (BB), L-buthionine-[S,R]-sulphox-imine (BSO), Coomassie brilliant blue R250, Dulbecco's Modified Eagle's Medium (DMEM, low glucose, without L-glutamine and phenol red), 5,5'-dithio-bis(2-nitrobenzoic acid) [DTNB], 7-ethoxycoumarin (7-EC), firefly lantern extract (FLE 250, 10 mg dried laterns/ml), gentamicin solution (10mg/ml), glucose-6-phosphate (G-6-P), glucose-6-phosphate dehydrogenase, L-glutamine, glutathione (GSH), glutathione disulphide (GSSG), glutathione reductase (230 unit/mg protein), hydrocortisone, 7-hydroxycoumarin (7-HC), insulin, MEM non-essential amino acid solution (100X), β-nicotinamide adenine dinucleotide (NADH, reduced form), β-nicotinamide adenine

dinucleotide phosphate (NADP), β-nicotinamide adenine dinucleotide phosphate (NADPH, reduced form), penicillin/streptomycin (5000 IU/ml & 5000 µg/ml), 7-pentoxyresorufin, phenobarbital (sodium salt), resorufin, 1,1,3,3-tetraethoxypropane, N,N,N′,N′-tetramethylethylenediamine (TEMED), 2-thiobarbituric acid (TBA), thymidine 5′-monophosphate p-nitrophenyl ester (PNPPT), triethanolamine, X-ray film were all purchased from Sigma Chemical Co. (Poole, Dorset, U.K).

Alanine aminotransferase kits were purchased from Boehringer Mannheim Biochemia Co. (U.K).

Acrylamide, potassium standard solution (potassium nitrate, 1000 ppm) were purchased from BDH (U.K).

Ecoscint™ H scintillation fluid was purchased from Mensura Technology Ltd. (Parbold, Wigan, U.K).

L-leucine [4,5-^{3}H] (specific activity: 77 Ci /mmole) was purchased from ICN Biomedicals Ltd.(Oxfordshire, U.K).

^{35}S-methionine (specific activity: 43 Ci / mg S) and Amplify™ were purchased from *Amersham* Life Science (Buckinghamshire, U.K).

Low molecular weight calibration kits for polyacrylamide gels were purchased from Pharmacia Biotech (U.S.A).

Tissue culture plates were purchased from Philip Harris Scientific Co. (Manchester, U.K).

All other chemicals were of reagent grade and from commercial sources.

2.2 Animals

Adult male Wistar rats (200-300 g) were used in all experiments. Animals were obtained from the breeding colonies at the Life Sciences Support Unit of Liverpool John Moores University and maintained in a relative humidity of 50% and in a 12 h light/12 h dark cycle at 22 °C. They were housed on wood shavings in NKP cages and allowed food (CRM1 Expanded diet, Special Diet Services Ltd., Witham, Essex, U.K) and water *ad libitum.*

For the bromobenzene toxicity experiments, mixed function oxygenases were induced by pretreating rats for four days with sodium phenobarbital by intraperitoneal injection at 100 mg/kg/day employing 0.9% saline (2 ml/kg) as the injection vehicle. Animals were sacrificed by cervical dislocation 24 h after the last administration.

2.3 General Description of The Krumdieck Tissue Slicer

The Krumdieck Tissue Slicer (MD 1100-A1, Alabama Research and Development Co., USA) is designed to rapidly prepare aseptic, thin slices of live tissue suitable for biochemical, pharmacological, toxicological and other *in vitro* studies. The slicer

mechanism operates submerged in a buffer solution selected by the operator as most appropriate in terms of composition, tonicity, pH, temperature, oxygenation and lubricating properties to maintain the viability of the tissue being sectioned. The instrument consists of three main components : the microtome assembly, the reservoir assembly and the electrical housing (Figures 2.1 and 2.2). The actual slicing is done by a rapidly oscillating disposable blade driven by a motor that also powers the impeller. The impeller establishes a circulating system of buffer that is used to gently carry the cut slices from the microtome to the glass trap. A speed-controlled second motor moves the tissue core past the oscillating blade and can be operated either intermittently to produce a single slice or continuously to produce slices at rates of up to 30/min.

The microtome/reservoir assembly was routinely placed in a dry oven and heated at 120 °C for 1 h for sterilisation and kept in a cold room at 4 °C for 24 h before use.

2.4 Preparation of The Slicing Buffer and Culture Media

The buffer which was used for the preparation of slices with the Krumdieck Tissue Slicer was a Krebs-Henseleit buffer of the following composition: 6.87 g of NaCl, 0.4 g of KCl, 0.14 g of $MgSO_4.7H_2O$, 0.1 g of NaH_2PO_4, 2.1 g of $NaHCO_3$, 0.37 g of $CaCl_2.2H_2O$ and 4.5 g of glucose per litre. The buffer was prepared by dissolving all of the salts except for $MgSO_4.7H_2O$ and $CaCl_2.2H_2O$ in dH_2O. These two salts were dissolved separately in 10 ml of dH_2O each and added to the above. The buffer was cooled to 4 °C and oxygenated for at least 1 h with 95% O_2/5% CO_2. The buffer was finally filter-sterilised using a filter sterilisation unit (with filling bell, 0.22 μm) and stored

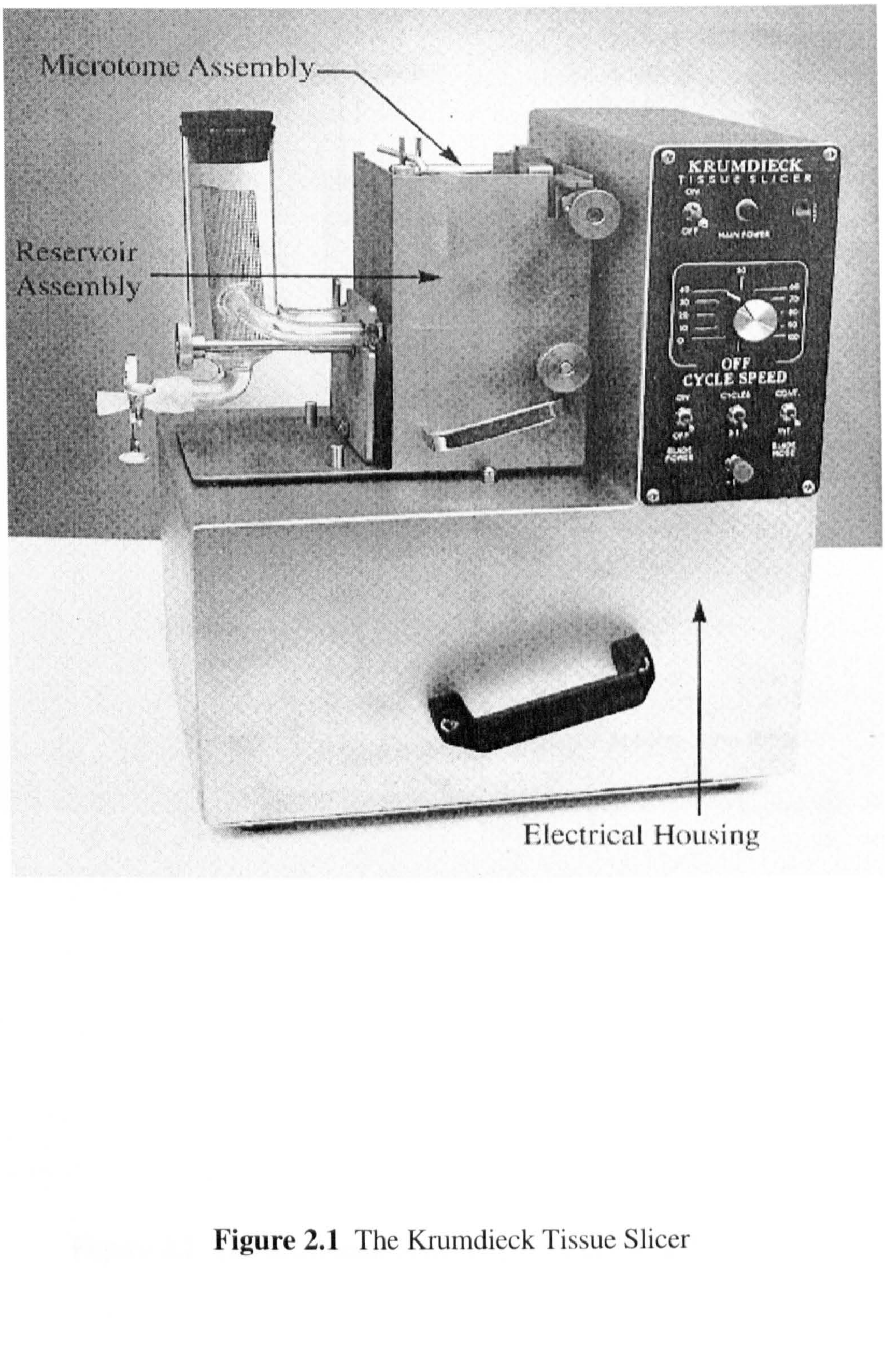


Figure 2.1 The Krumdieck Tissue Slicer

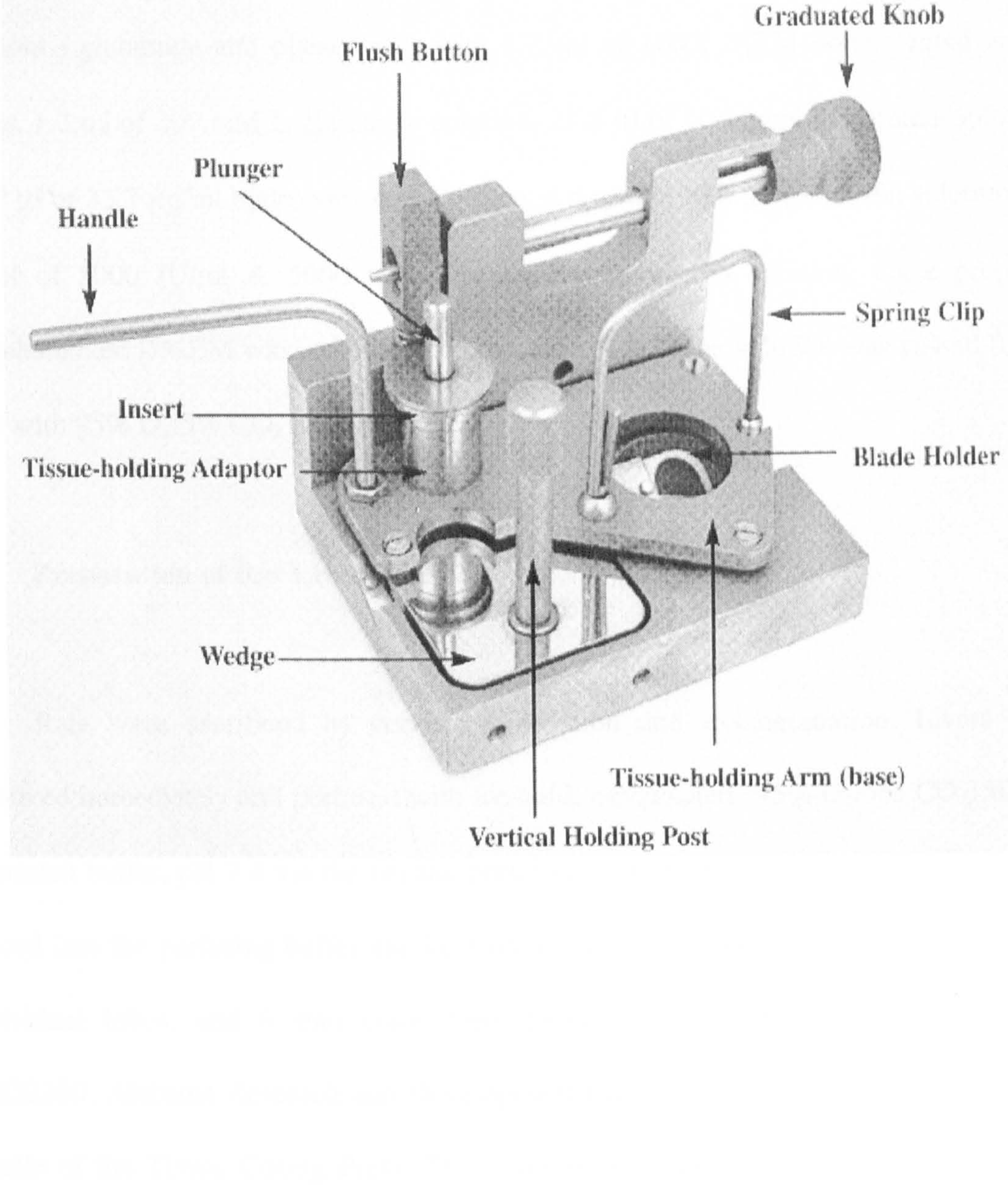


Figure 2.2 The Microtome Assembly of Krumdieck Tissue Slicer

in sterilised glass bottles. The pH of the buffer was adjusted to 7.4 with NaOH just before use.

Culture medium was prepared by supplementing 100 ml of DMEM (low glucose, without L-glutamine and phenol red) with 1.2 ml of 100X MEM non-essential amino acids, 1.2 ml of 200 mM L-glutamine solution, 22.8 µl of 10 mg/ml gentamicin solution, 25.2 µl of 33.7 µg/ml hydrocortisone solution, 4.5 µl of 130.8 µg/ml insulin solution and 1 ml of 5000 IU/ml & 5000 µg/ml penicilin/streptomycin solution. Once prepared supplemented DMEM was stored for up to 24 h at 4 °C. The solution was gassed for 10 min with 95% O_2:5% CO_2 prior to use.

2.5 Preparation of Rat Liver Slices

Rats were sacrificed by cervical dislocation and exsanguination. Livers were removed immediately and perfused with ice-cold, oxygenated (95% O_2/5% CO_2) Krebs-Henseleit buffer, pH 7.4 via the hepatic portal vein to remove blood. The liver was then placed into the perfusing buffer and kept on ice. Rat livers were carefully dissected into individual lobes, and 8 mm cores were produced using the Tissue coring Press (MD2300, Alabama Research and Development Co., USA) [Figure 2.3] by turning the handle of the Tissue Coring Press. The cores were immediately placed in a Petri dish containing ice-cold Krebs-Henseleit buffer, pH 7.4 and maintained on ice until slicing.

Rat liver slices were made using a Krumdieck Tissue Slicer from individual tissue cores. The entire slicing process was performed in ice-cold, oxygenated Krebs-Henseleit

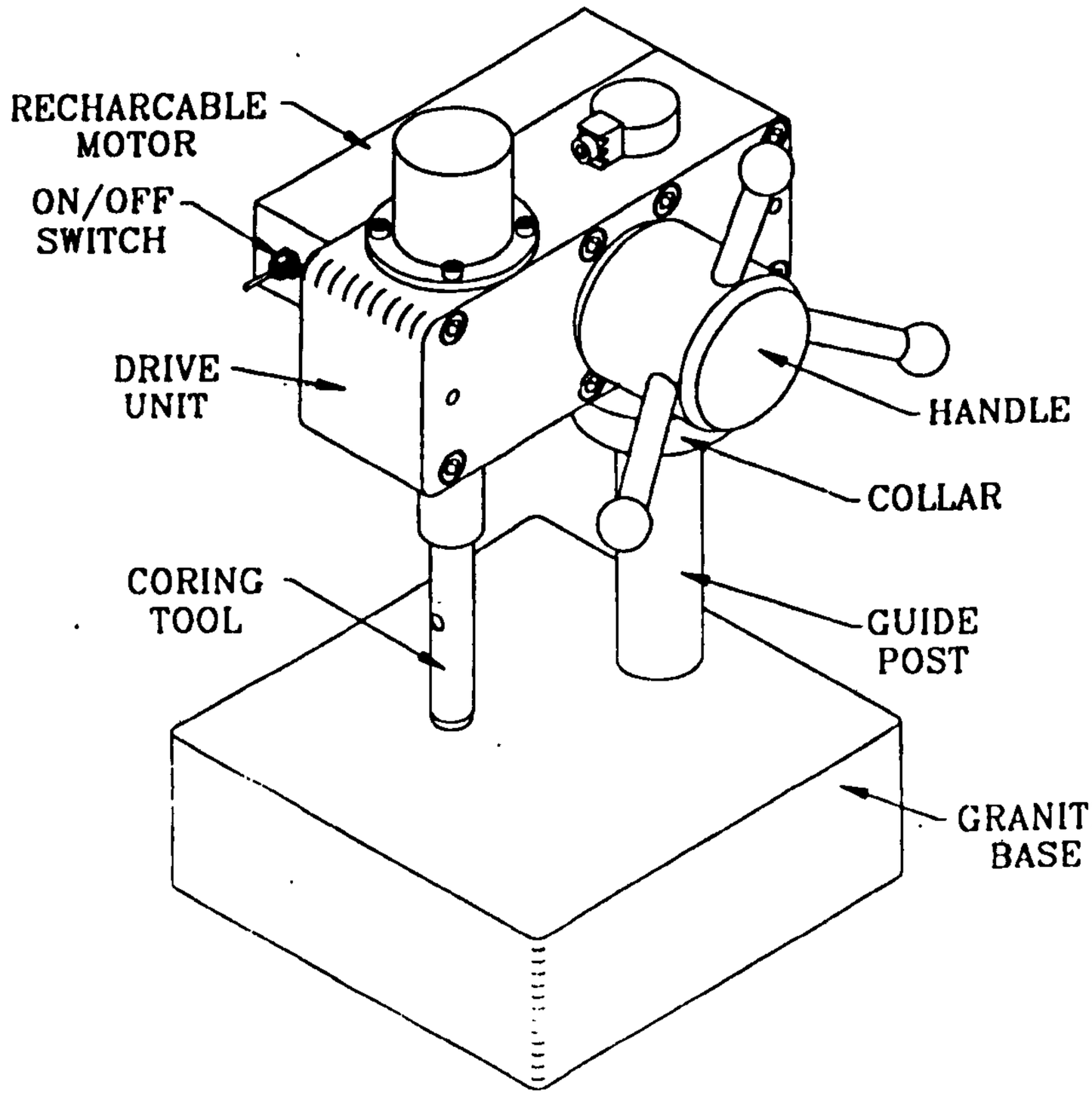


Figure 2.3 The Tissue Coring Press

buffer, pH 7.4. The tissue core was placed in the insert and the plunger with 3 weighted washers placed on top of the tissue core. The slice thickness (250 ± 20 μm) was set by turning the graduated knob. The first slice produced from each core was discarded as it may have the liver capsule as one side rather than exposed cells. The freshly-sectioned slices were collected from the glass trap and stored in ice-cold buffer until incubation. Slices with a diameter of 8 mm and thickness of 250 ± 20 μm had a wet weight of 13-16 mg and contained 0.8-1.0 mg of protein.

2.6 Development of a Simple Incubation System for Rat Liver Slices

Slices were incubated as described by Connors *et al.* (1990) with some modifications. Prior to incubation, the slices were washed twice in supplemented DMEM. Individual slices were transferred into a 24-well plastic tissue culture plate filled with 0.5 ml of supplemented DMEM per well. Plates were put on an orbital shaker oscillating at 60 rpm and contained within an incubator at 37 °C with saturated humidity and an atmosphere of 95% air/5% CO_2. All slices were preincubated for 30 min at 37 °C before any experiments were undertaken.

2.7 Preparation of Rat Liver Microsomes

Rat liver microsomes were prepared by differential centrifugation as described by Lake (1987). Rats were killed by cervical dislocation, the livers rapidly excised and washed in ice-cold 154 mM KCl containing 50 mM Tris-HCl, pH 7.4. To form a 20% homogenate, livers were weighed , finely cut up using scissors and then homogenised in

4 volumes of ice-cold 154 mM KCl containing 50 mM Tris-HCl, pH 7.4 by 10 up-and-down stokes in a Potter-Elvehjem homogeniser with the motor-driven teflon pestle rotating at 500 rpm. The liver homogenate was then transferred into pre-cooled ultracentrifuge tubes, accurately balanced and capped, and centrifuged at 10,000 g for 20 min at 4 °C in a Beckman L8-80 ultracentrifuge. The resultant post-mitochondrial supernatant was transferred to fresh pre-cooled ultracentrifuge tubes (balanced and capped) and centrifuged at 105,000 g for 1 h at 4 °C. The supernatant was discarded and the microsomal pellet was resuspended in 20 ml of fresh homogenising buffer using a hand-held Potter-Elvehjem homogeniser and then recentrifuged at 105,000 g for 1 h at 4 °C. The supernatant was discarded and the washed microsomal pellet was resuspended by hand in homogenising buffer to a protein concentration of 5-10 mg/ml. The microsomes were used on the day of preparation.

The effects of AGE and SAC *in vitro* upon ECOD and PROD activities were determined by preincubating microsomes at 0 °C or 37 °C for 1 h with AGE at final concentrations of up to 5% (v/v) or with SAC at final concentrations of up to 1 mM prior to assay.

The effects of AGE *in vivo* upon ECOD and PROD activities were determined by pretreating animals orally with AGE for 7 days as described in Section 6.1. The liver was then excised and microsomes were prepared.

2.8 Biochemical Indicators of Slice Viability

2.8.1 Total protein assay

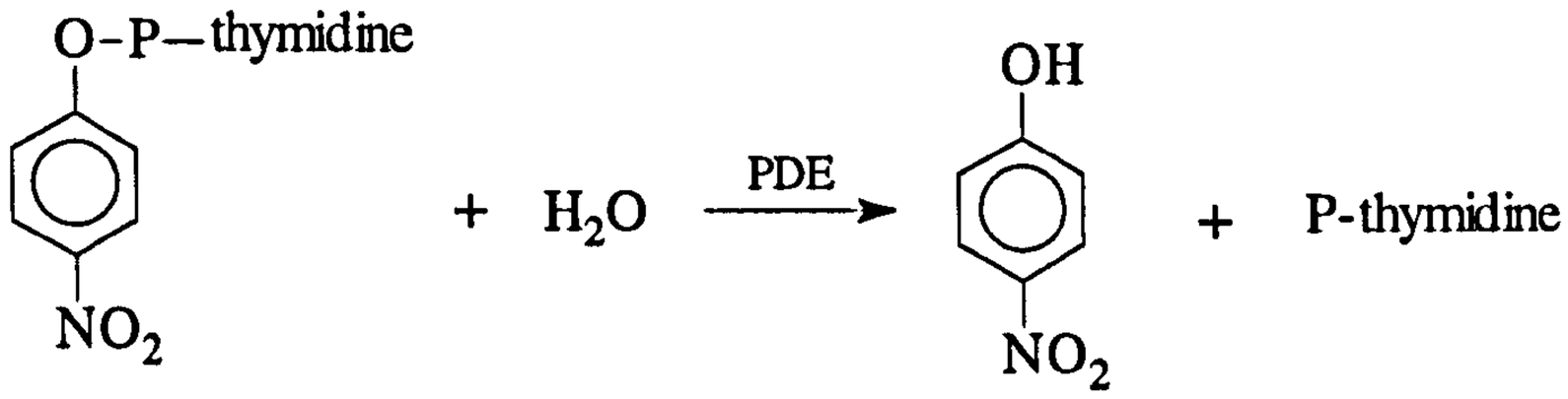
Following the appropriate times in culture, slices were blotted dry, placed in 0.5 ml of fresh medium and homogenised in a glass Dounce-type mini-homogeniser (Jencons Scientific Ltd., Leighton Buzzard, U.K). The protein content of the resultant homogenate was determined according to Winterbourne (1993). Whatman 3MM filter paper was marked in pencil with a grid of 1 cm^2 squares. Aliquots (4 µl) of homogenate were spotted into the centre of the squares on the sheet of Whatman 3MM and the filter paper was immersed in 10% TCA for 15 min. The sheet was transferred to a bath of working dye solution [0.04% (w/v) Coomassie blue, 25% (v/v) ethanol, and 12% (v/v) acetic acid], and stained for 1 h. Then the sheet was put in 100 ml of destain solution [10% (v/v) ethanol, 5% (v/v) acetic acid] for 10 min for each of three times and finally dried at room temperature or in an oven at 80 °C. The grid was cut into its component squares and each square placed in a test tube. The bound dye was eluted by addition of 1 ml of 1 M potassium acetate in 70% ethanol and gentle mixing for 1 h. The absorbance was measured at 590 nm on a spectrophotometer (LKB Biochem, Novaspec II). Protein concentrations were determined by interpolation of the linear relationship between absorbance and standard protein mass. The protein standard curve (Appendix I, Figure 1) was generated using a 1 mg/ml solution of bovine serum albumin made up in dH_2O.

Protein content in the culture medium was also measured by the above method. Results are expressed as % protein release and were calculated as follows:

$$\% \text{ release} = \frac{\text{protein in medium}}{\text{protein in slice} + \text{protein in medium}} \times 100$$

2.8.2 Alkaline phosphodiesterase assay

Alkaline phosphodiesterase 1 (EC 3.1.4.1, PDE) was measured by the methods of Brightwell & Tappell (1968) and Trams & Lauter (1974) based upon the following reaction:



thymidine 5'-monophosphate	*p*-nitrophenol
p-nitrophenyl (PNPPT)	(yellow colour)

Following appropriate times in culture, slice homogenates were prepared as described in Section 2.8.1 and, together with the relevant culture medium, were used for assay. To each well of a 96 well microtitre plate, the following were added: 133 µl of 50 mM glycine (pH 9.7), 13 µl of 150 mM $MgCl_2$ and 40 µl of sample (homogenate or medium). The substrate (13 µl of 15 mM PNPPT) was then added and after a 1 min lag period, the change in absorbance was determined at 405 nm at 10 min intervals for 40

min in a Titertek Multiskan® MCC/340 Platereader. The % PDE release was calculated by the method given in Section 2.8.1.

2.8.3 Alanine aminotransferase assay

L-alanine aminotransferase (EC 2.6.1.2, ALT) activity was assayed by a slight modification of the method of Segal *et al.* (1970) based upon the following linked reactions:

$$\alpha\text{-oxoglutarate} + \text{L-alanine} \xrightleftharpoons{\text{ALT}} \text{L-glutamate} + \text{pyruvate}$$

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \xrightleftharpoons{\text{LDH}} \text{lactate} + \text{NAD}^+$$

To make the assay buffer one reagent tablet was dissolved in one bottle of reagent solution of an ALT Kit. The resultant solution contained 80 mM phosphate buffer, pH 7.4, 800 mM L-alanine, ≥ 1.2 U/ml LDH, 0.18 mM NADH and 18 mM α-oxoglutarate. To each well of a 96-well microtitre plate was added 200 µl of assay buffer and 20 µl of sample (homogenate or media). The change in absorbance was determined at 340 nm at 10 min intervals for 40 min in a Titertek Multiskan® MCC/340 Platereader. The % ALT release was calculated by the method given in Section 2.8.1.

2.8.4 Lactate dehydrogenase assay

Lactate dehydrogenase (EC 1.1.1.27, LDH) activity was assayed by the method of Stolzenbach (1966) based upon the following reaction:

$$\text{lactate} + NAD^+ \overset{LDH}{\rightleftharpoons} \text{pyruvate} + NADH + H^+$$

In a cuvette, 2.5 ml of 120 mM potassium phosphate buffer, pH 7.4 was added to 200 µl of 10 mM pyruvate and 200 µl of 2 mM NADH. The cuvette was placed in a CE 292 Digital Ultraviolet spectrophotometer and when the absorbance at 340 nm was steady, 100 µl of sample (homogenate or medium) was added and the change in O.D. recorded over 1 min. The % LDH release was calculated by the same method given in Section 2.8.1.

2.8.5 K^+ assay

The intracellular K^+ content of slices was measured by means of a flame photometer according to the method of Smith *et al.* (1987).

Following the appropriate times in culture, slices were rinsed in normal saline (0.9% NaCl) in order to eliminate external potassium from the culture medium. Slices were blotted dry, placed into 1 ml of distilled water and homogenised in a glass mini-homogeniser. An aliquot of homogenate (0.5 ml) was transferred to a microfuge tube containing an equal volume of 2.75 % (v/v) perchloric acid (PCA) to precipitate protein.

This mixture was vortexed and then centrifuged at 10,000 g for 10 min in a microfuge (MSE Micro Centaur). The supernatant fraction was assayed for K^+ using a Jenway model PFP7 flame photometer based on a standard curve (Appendix I, Figure 3) which ranged from 0 to 512 μM (0-20 ppm). The protein content of slice homogenates was determined by the Coomassie blue staining method as described in Section 2.8.1. K^+ concentrations were calculated from the standard curve by linear regression analysis and are expressed as nmol of K^+/mg of protein.

2.8.6 ATP assay

The ATP content of slice homogenates was measured by means of the firefly luciferase enzyme assay of Holm-Hansen *et al.* (1978) with slight modifications. This assay depends on the detection of the bioluminescence produced by the luciferin extract of firefly tails in the presence of ATP according to the following reaction:

$$ATP + LH_2 + O_2 \xrightarrow{FL} AMP + PPi + CO_2 + P + hv$$

where FL = firefly luciferase, LH_2 = reduced luciferin (4,5-dihydro-2-[6-hydroxy-2-benzothiazolyl]-4-thiazolecarboxylic acid), PPi = inorganic pyrophosphate, P = product (oxidased luciferin), hv = light.

After the appropriate times in culture, slices were homogenised in 900 μl of 75 mM potassium phosphate buffer, pH 7.4 containing 100 μl of 20% (v/v) PCA. The resultant homogenate was centrifuged at 10,000 g for 10 min in a MSE Micro Centaur microfuge. A portion of the supernatant (500 μl) was removed and neutralised by the

addition of 72 µl of 0.5 M triethanolamine in 2 M KOH. ATP content was assayed by mixing 50 µl of the neutralised supernatant with 420 µl of 75 mM potassium phosphate buffer, pH 7.4 containing 15 mM $MgCl_2$ and 30 µl of firefly lantern extract (FLE) in a lunimometer tube. Luminescence was measured immediately in a Bio-orbit model 1251 luminometer (Labsystem, U.K). The homogenate ATP concentrations were calculated from the standard curve in the range 0-20 µM (Appendix I, Figure 4). Results are expressed as nmol of ATP/mg of protein.

2.8.7 Protein synthesis assay

Slice protein synthesis was measured as incorporation of ^{3}H-leucine into acid precipitable protein (Smith *et al.*1987).

The slices were incubated in the presence of 0.3 µCi/ml ^{3}H-leucine. The specific activity of ^{3}H-leucine was 77 Ci/mmol; thus 0.3 µCi/ml is equivalent to 3.9 nM ^{3}H-leucine, which is insignificant compared to concentration of unlabelled leucine in supplemented DMEM (0.8 mM).

After the appropriate times in culture, slices were removed and immediately washed in two changes of ice-cold Krebs-Henseleit buffer and homogenised in 1 ml of 1 M KOH. Aliquots (4 µl) of the homogenates were taken for protein determination as described in Section 2.8.1. Following the addition of an equal volume of 1.5 M acetic acid, the remaining homogenate was centrifuged at 10,000 g for 10 min in a MSE Micro Centaur microfuge. The pellets were washed twice in 2 M acetic acid and then dissolved

in 0.5 ml of 0.5 M NaOH. The incorporation of ^{3}H-leucine into acid precipitable protein was determined by counting a 0.4 ml aliquot of the dissolved pellets after neutralisation with 125 µl of 2M HCl, in 4.5 ml of Ecoscint H scintillation fluid in a liquid scintillation counter (LS 6500 Multi-purpose Scintillation system, Beckman instruments, Inc., California, U.S.A). Results are expressed as dpm /mg protein.

2.8.8 Protein secretion assay

Secretion of acid-precipitable radiolabelled protein into the culture medium of liver slices was measured over 24 h (Smith, 1986). At the appropriate times, 0.5 ml aliquots of the culture medium were taken and mixed with 0.2 ml of ice-cold 10 % (v/v) PCA. The denatured extracellular proteins were collected by centrifugation at 10,000 g for 5 min and washed by resuspending the precipitate in 1 ml of ice-cold 20% (v/v) PCA. The pellet was washed two more times in this manner and finally was dissolved in 0.5 ml of 0.5 M NaOH. The incorporation of ^{3}H-leucine into secreted protein was then determined as described in Section 2.8.7. Data are expressed as dpm per ml of culture medium.

2.8.9 Slice non-protein sulphydryl content

Non-protein sulphydryl (NPSH) content of liver slices was measured using the method of Ellman (Beutler *et al.* 1963; Sedlak and Lindsay, 1968). This is a simple spectrophotometric assay in which dithio-bis-nitrobenzoic acid (DTNB) gives a coloured product when bound to free sulphydryl groups.

Slices were removed from culture at the appropriate times, washed with ice-cold saline and homogenised in 1 ml of ice-cold ethanol, 24 mM potassium phosphate buffer, pH 5.5 containing 0.1 mM EDTA (3:1, v/v) and centrifuged at 10,000 g for 10 min in a MSE Micro Centaur microfuge. The supernatant was decanted and used in the assay. The protein pellet was resuspended in 1 ml of 1 M NaOH and assayed for protein content by the Coomassie blue staining method as described in Section 2.8.1. To each well of a 96-well plate was added 75 µl of the supernatant, 187.5 µl of 0.3 M sodium phosphate buffer, pH 7.4 containing 1 mM EDTA and 37.5 µl of 1 mM DTNB in 1 % tri-sodium citrate. Absorbances were read within 5 min at 412 nm in a Titertek Multiskan® MCC/340 platereader and NPSH contents were calculated using glutathione as standard in the range 0-40 µM (Appendix I, Figure 5). Data are expressed as nmols NPSH/mg of protein.

2.8.10 Slice glutathione content

The total glutathione (i.e reduced glutathione [GSH] and glutathione disulphide [GSSG]) content of slices was determined using the DTNB-GSSG reductase recycling method (Anderson, 1985). Since GSSG represents < 0.5% of total cellular glutathione, this provides a specific assay of GSH according to the following reactions:

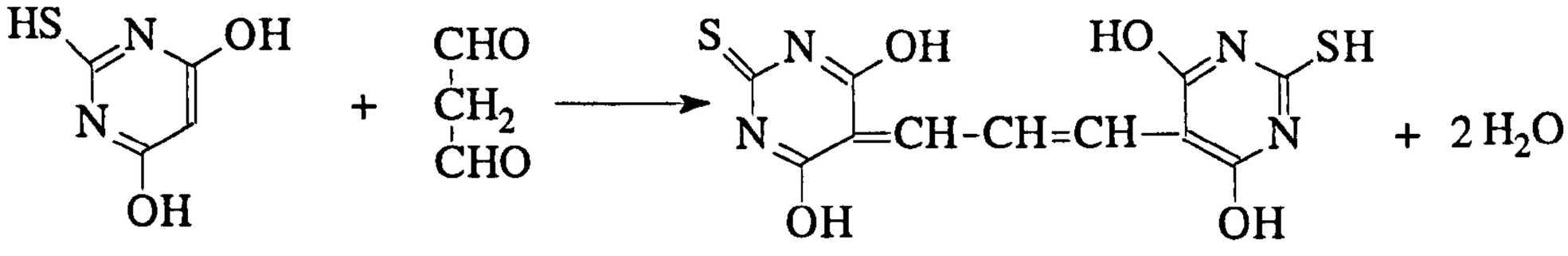
$$2\,GSH + DTNB \longrightarrow GSSG + TNB$$

$$GSSG + NADPH + H^{+} \xrightarrow[\text{Reductase}]{GSSG} 2\,GSH + NADP^{+}$$

At the appropriate times, slices were removed and homogenised in 0.1 ml of 0.1 M sodium phosphate buffer, pH 7.4. To 50 µl of the homogenate, an equal volume of 4% (v/v) 5-sulphosalicylic acid was added and then centrifuged at 10,000 g for 10 min in a MSE Micro Centaur microfuge. The supernatant was assayed for total GSH. In a cuvette, 700 µl of 0.3 mM NADPH in 143 mM sodium phosphate buffer, pH 7.5 containing 6.3 mM EDTA was added to 100 µl of 6 mM DTNB in the same buffer in which NADPH dissolved, 175 µl of dH_2O and 25 µl of the homogenate supernatant. The cuvette was placed in a CE 292 Digital Ultraviolet spectrophotometer. GSSG-reductase (8 µl of 322 unit/ml) was added with mixing to initiate the reaction. The increase in absorbance at 412 nm was registered on a chart recorder for up to 1 min. The amount of GSH was determined from a standard curve (Appendix I, Figure 6) in the range 0 - 3 nmol of GSH. Results are expressed as nmols GSH per mg protein.

2.8.11 Thiobarbituric acid reacting substances formation assay

Malondialdehyde (MDA) formed from the breakdown of polyunsaturated fatty acids serves as a convenient index for determining the extent of the peroxidation reaction. MDA has been identified as the product of lipid peroxidation that reacts with thiobarbituric acid (TBA) to give a pink coloured product at 532 nm. Hence MDA was measured as thiobarbituric acid reacting substances (TBARS). The formation of TBARS in the culture medium was used as a measure of lipid peroxidation (Buege and Aust, 1978) based upon the following reaction:



TBA MDA pink product

After incubation of slices, 2 ml of TBA reagent containing 0.375% (w/v) TBA, 15% (w/v) TCA in 0.25 M HCl was added to 0.5 ml of culture medium. The reaction mixture was heated in a boiling water bath for 10 min, cooled to room temperature and centrifuged at 2000 g for 10 min in Beckman model J-6B centrifuge. TBARS concentration in the supernatant were determined at 532 nm in a spectrophotometer (LKB Biochem, Novaspec II) and calculated by using a standard curve (Appendix I, Figure 2) in the range of 0 -5 μM of MDA. MDA is unstable and must therefore be prepared immediately before use by hydrolysing 1,1,3,3-tetraethoxypropane to MDA in 1% H_2SO_4 for 2 h. Results are expressed as nmols MDA produced per 100 mg of protein.

2.8.12 7-Ethoxycoumarin O-deethylase activity assay

7-Ethoxycoumarin O-deethylase (ECOD) activity was measured in rat liver slices and microsomes by a fluorimetric method essentially as described by Lake (1987). The O-deethylation of 7-ethoxycoumarin (7-EC) results in the production of 7-hydroxycoumarin (7-HC) which is readily estimated fluorimetrically.

Slices were homogenised in 0.5 ml of 50 mM Tris-HCl buffer, pH 7.8. The reaction mixture contained up to 75 µl of slice homogenate or microsomes (equivalent to approximately up to 500 µg of protein) and 50 µl of a NADPH generating system (final concentrations: 4.5 mM G-6-P, 0.17 IU/ml glucose-6-phosphate dehydrogenase, 0.19 mM $NADP^+$ and 4.8 mM $MgCl_2$) made up to a final volume of 0.5 ml with 50 mM Tris-HCl buffer, pH 7.8. The reaction was initiated by the addition of 125 µl of 2 mM 7-EC in 50 mM Tris-HCl buffer, pH 7.8 and the samples were incubated at 37 °C for 10 min. The reaction was terminated by the addition of 0.25 ml of 5% (w/v) $ZnSO_4$ and 0.25 ml of saturated $Ba(OH)_2$ and protein precipitated by centrifugation at 10,000 g for 10 min in a MSE Micro Centaur microfuge. Deproteinised supernatant (0.8 ml) was added to 2.4 ml of 0.5 M glycine-NaOH buffer, pH 10.5 and the fluorescence was measured in a Perkin-Elmer LS 50B spectrofluorimeter, using an excitation wavelength of 380 nm and an emission wavelength of 452 nm. ECOD activity was calculated by reference to a standard curve in the range 0 - 6 nmol of 7-HC (Appendix I, Figure 7). ECOD activity in the slices or microsomes is expressed as nmol/min/mg slice or microsomal protein.

Linearity of reaction rates was examined for microsomal preparations. It was found that volumes of up to 50 µl of microsomes (equivalent to approximately 300 µg of protein) from control rats and up to 25 µl (equivalent to approximately 150 µg of protein) from phenobarbital-induced rats gave linear reaction rates. The experiments presented in Table 5.1, which investigated the effect of AGE on ECOD activity, were performed using 75 µl of microsomes in the assay. All other ECOD assays reported in this thesis were carried out using non-limiting volumes of microsomes.

2.8.13 7-Pentoxyresorufin O-depentylase activity assay

7-Pentoxyresorufin O-depentylase (PROD) activity was measured in rat liver microsomes essentially as described by Burk *et al.*(1985) and Lubet *et al.* (1985). The microsomes were prepared as described in Section 2.7. The reaction mixture contained 100 µl of microsomes, 100 µl of 50 µM 7-pentoxyresorufin dissolved in DMSO and 1.76 ml of 100 mM Tris-HCl buffer, pH 7.6. The mixture was equilibrated for 1 min at room temperature and the reaction started by the addition of 40 µl of 25 mM NADPH. The increasing fluorescence was recorded for up to 10 min in a Perkin-Elmer LS 50B spectrofluorimeter using an excitation wavelength of 510 nm and an emission wavelength of 586 nm. The PROD activity was calculated by comparison to a standard curve in the range 0- 0.5 nmol of resorufin (Appendix I, Figure 8) and results are expressed as pmol/min/mg of microsomal protein.

2.8.14 SDS-PAGE and autoradiography

The proteins in cultured slices were separated by SDS-PAGE according to the method of Dunn and Bradd (1993).

^{35}S-Methionine was added to medium prior to culture (50 µCi/ml). After appropriate times in culture, slices were removed and immediately washed in two changes of ice-cold Krebs-Henseleit buffer and homogenised in 300 µl of 0.5% (w/v) sodium dodecyl sulphate (SDS). One volume of the homogenate was added to 4 volumes of 100% (v/v) ethanol, cooled to -70 °C for 30 min and centrifuged at 10,000 g for 10

min (4 °C) in a MSE Micro Centaur microfuge. The pellets were washed twice in 70% (v/v) ethanol and dissolved in 100 µl sample buffer [0.125 M Tris-HCl, pH 6.8, 4% (w/v) SDS, 0.002% (w/v) Bromophenol blue, 20% (v/v) Glycerol, 10% (v/v) β-mercaptoethanol], vortexed and heated at 100 °C for 5 min.

To prepare a 15% separating gel, 49.95 ml of 30% (w/v) stock acrylamide solution, 25 ml of 1.5 M Tris-HCl (pH 8.8), 1 ml of 10% SDS, 150 µl of 10% (w/v) ammonium persulphate (AP), 50 µl of TEMED and 23.85 ml of dH_2O were mixed and poured in to the gel cassettes (20 × 20 cm). The gel was left to polymerise at room temperature for 1 h. To prepare the 3% (w/v) stacking gel, 3 ml of 30% (w/v) acrylamide stock, 3.75 ml of 1M Tris- HCl (pH 6.8), 22.79 ml of dH_2O, 135 µl of 10% (w/v) AP, 375 µl of 10% (v/v) SDS and 30 µl of TEMED were mixed and poured onto the top of the polymerised separating gel. The appropriate sample well-forming comb was placed at the top. After 1 h, when the stacking gel should have polymerised, the sample combs were removed from the gels and the gels installed in the electrophoresis apparatus. The electrode chambers were filled with the reservoir buffer solution (0.192 M Glycine, 0.025 M Tris, 0.1% SDS). Samples and molecular weight standards (30 µl) were loaded into the sample wells using micropipettes fitted with narrow-ended tips. The apparatus was connected to a power pack and the gels were run under constant current conditions of 30 mA/gel for up to 8 h. After protein separation, the gels were removed from the cassettes, stained with 0.16 % (w/v) Coomassie brilliant blue R-250 for 15 min and destained with 20 % (v/v) methanol and 7.5 % (v/v) acetic acid. The gels were immersed in 50 ml of Amplify™ for 30 min, dried onto a filter paper at 80 °C for 1 h and subsequently exposed to a X-ray film in a X-ray box for three days. The film was

developed in an automatic film developer (Kodak M35A X-OMAT) and used for analysis.

2.8.15 Morphological investigation of slices: electron microscopy

Electron microscopy of liver slices was performed essentially as described by Beamand (1993).

After the appropriate times in culture, liver slices were removed and fixed in 0.5% (w/v) glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4 for 1 h. The slices were rinsed in 0.1 M sodium cacodylate buffer, pH 7.4 for six times and post-fixed with 2% (w/v) osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4 at room temperature for 1 h and at 4 °C for another 3 h. The slices were stained in 2% (w/v) aqueous uranyl acetate at room temperature for 2 h and washed with dH_2O for three times. The slices were dehydrated in 30%, 50%, 70%, 90% and 100% (v/v) graded ethanol for 10 min at each concentration. After embedding in Epon Araldite resin overnight, ultrathin sections of selected areas were cut and picked up on uncoated copper grids and stained with 1% (v/v) uranyl acetate for 10 min followed by lead citrate (Reynolds, 1963). The sections were examined using a JEM-1200EX Electron Microscope (JEOL Ltd. Tokyo Japan).

2.9 Presentation of Results

All values are presented as mean ± SD from at least 3 experiments. All statistical analyses were carried out using an unpaired two-tailed Student's *t-test* and differences were considered significant at $P < 0.05$.

CHAPTER 3

VIABILITY OF CULTURED LIVER SLICES

3.1 Results

3.1.1 Incubation conditions for maintenance of liver slices

Slices were incubated individually at 37 °C under an atmosphere of 95% air/5% CO_2 in 24-well plates at a rotation speed of 60 rpm. At this speed, the culture medium was mixed moderately, the slices floated and moved smoothly and leakage of all three enzymes measured was modest (Table 3.1). Increased loss of intracellular and plasma membrane enzymes occurred when the rotation speed was increased to 90 rpm (Table 3.1). Under these conditions, slices could be kept viable for up to 24 h in culture and thus all future work used 60 rpm.

Table 3.1 Effect of shaking speed upon viability of rat liver slices

shaking speed	% LDH	% ALT	% PDE
60 rpm	10.0 ± 1.5	5.0 ± 1.0	2.1 ± 0.7
90 rpm	29.0 ± 3.2	15.9 ± 1.8	6.9 ± 1.5

Rat liver slices were incubated at 37 °C for 4 h on an orbital shaker at either 60 or 90 rpm. LDH, ALT and PDE leakage from cultured liver slices is expressed as % release into the culture medium. Values are means ± SD from three animals.

3.1.2 Release of total protein and marker enzymes from cultured liver slices

The % release of total protein over 24 h is shown in Figure 3.1A. Release of protein increased gradually over the first 8 h and by 24 h was still < 25%. Between 4 and 8 h, protein release increased from 5% to 16%. These results are very similar to those reported by Beamand *et al.* (1993).

Release of the plasma membrane enzyme PDE from cultured liver slices is shown in Figure 3.1B. After 24 h in culture only 4% of the enzyme was released into culture medium. In contrast, much large amounts of the cytosolic enzyme LDH were released (Figure 3.2B). Leakage of this enzyme was under 10% for up to 4 h in culture, but then increased rapidly such that by 24 h, nearly 60% of slice LDH was released into the medium. Leakage of the cytosolic and mitochondrial enzyme ALT was intermediate between that of PDE and LDH (Figure 3.2A). ALT release increased from approximately 5% after incubation for 4 h to nearly 20% after incubation for 24 h.

3.1.3 K^+ and ATP content of cultured liver slices

Figure 3.3A shows the K^+ content of cultured liver slices over 24 h. Following an initial recovery period, the K^+ content of cultured slices rose to a maximum level by 4 h. Between 4 and 8 h, slice K^+ content decreased to approximately 70% of this. At 8 h, the K^+ content corresponded to that in freshly isolated slices and then was maintained for the rest of the incubation period.

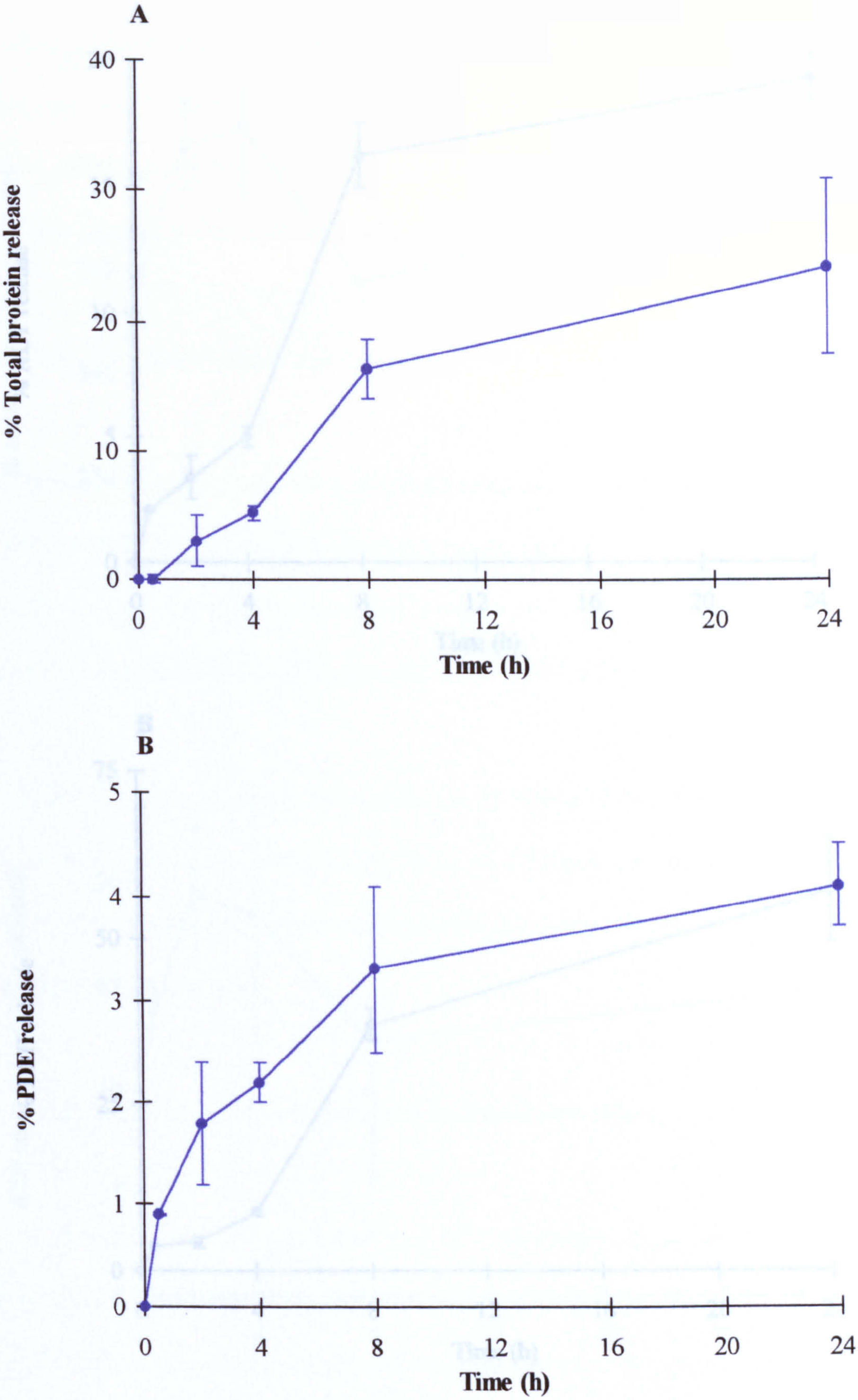


Figure 3.1 Release of total protein (A) and PDE (B) from rat liver slices over 24 h of incubation. Values are means ± SD from 5 animals.

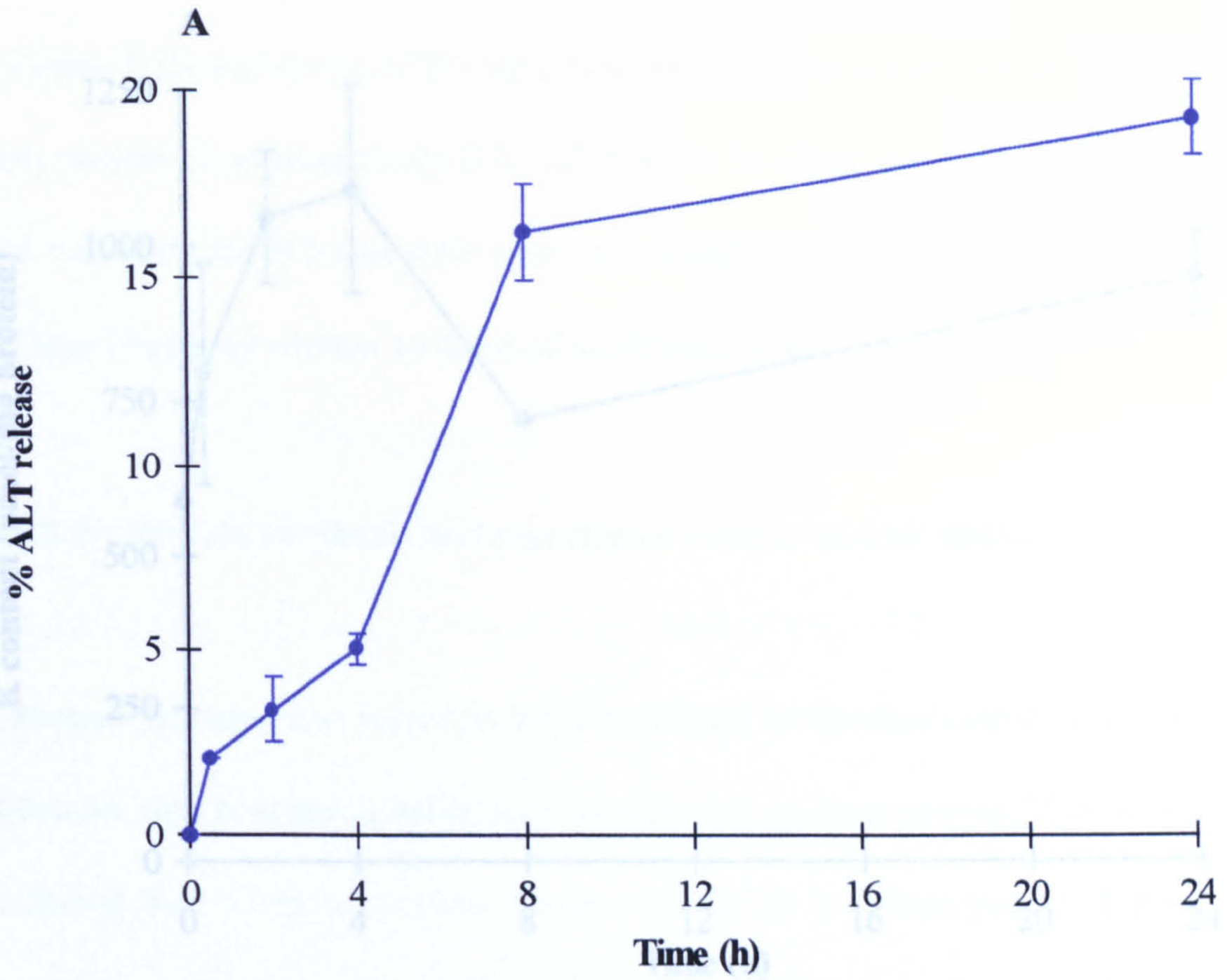


Figure 3.2 Release of ALT (A) and LDH (B) from rat liver slices over 24 h of incubation. Values are means ± SD from 5 animals.

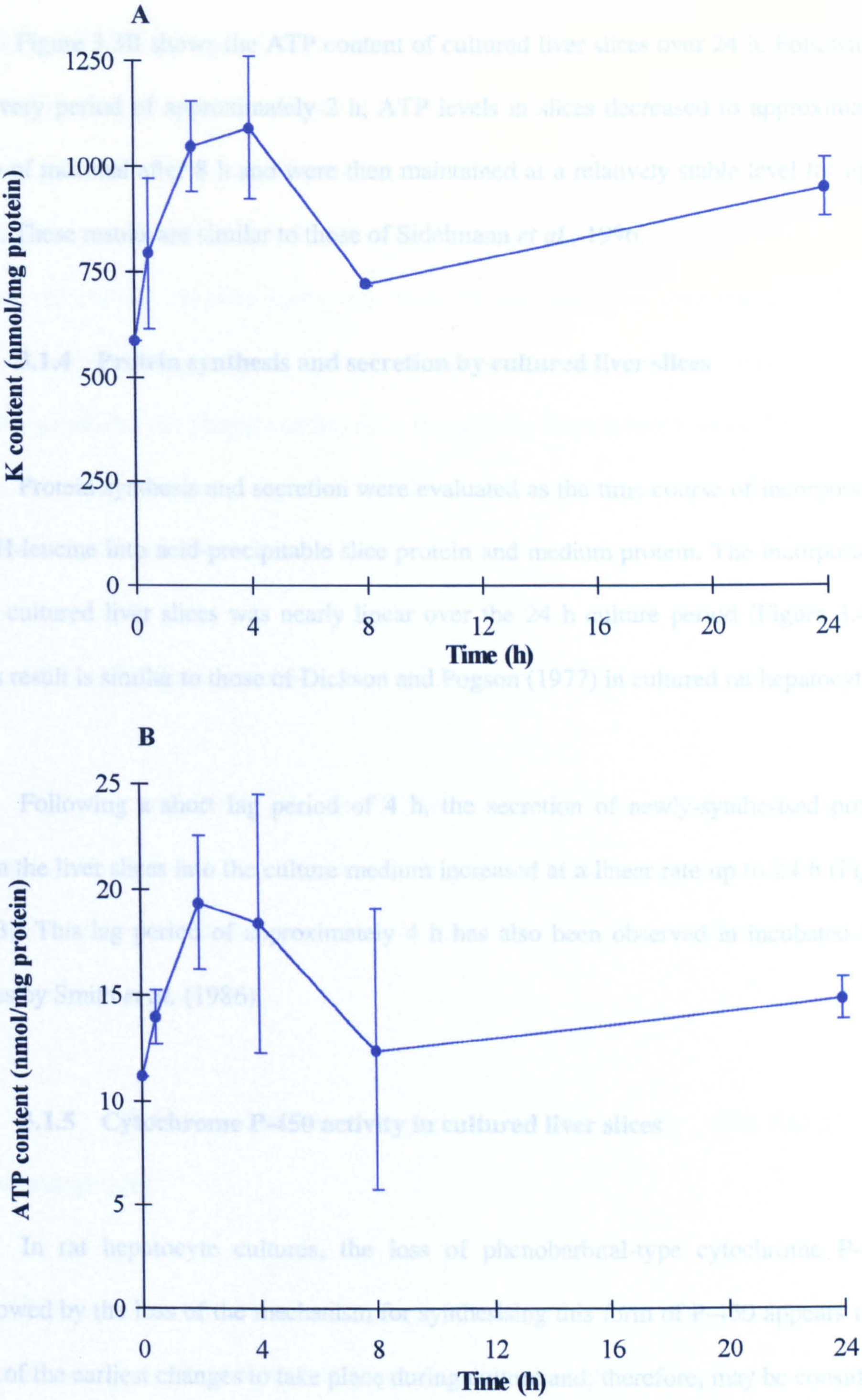


Figure 3.3 K^+ (A) and ATP (B) content of rat liver slices over 24 h of incubation. Values are means ± SD from at least 3 animals. The zero time values for both indices were determined on slices after 30 min of preincubation at 37 °C.

Figure 3.3B shows the ATP content of cultured liver slices over 24 h. Following a recovery period of approximately 2 h, ATP levels in slices decreased to approximately 65% of maximal after 8 h and were then maintained at a relatively stable level for up to 24 h. These results are similar to those of Sidelmann *et al.*, 1996.

3.1.4 Protein synthesis and secretion by cultured liver slices

Protein synthesis and secretion were evaluated as the time course of incorporation of ^{3}H-leucine into acid-precipitable slice protein and medium protein. The incorporation into cultured liver slices was nearly linear over the 24 h culture period (Figure 3.4A). This result is similar to those of Dickson and Pogson (1977) in cultured rat hepatocytes.

Following a short lag period of 4 h, the secretion of newly-synthesised protein from the liver slices into the culture medium increased at a linear rate up to 24 h (Figure 3.4B). This lag period of approximately 4 h has also been observed in incubated liver slices by Smith *et al.* (1986).

3.1.5 Cytochrome P-450 activity in cultured liver slices

In rat hepatocyte cultures, the loss of phenobarbital-type cytochrome P-450, followed by the loss of the mechanism for synthesising this form of P-450 appears to be one of the earliest changes to take place during culture and, therefore, may be considered an important viability parameter (Bridges, 1981). As an index of mixed function oxygenase activity, the O-deethylation of 7-EC was measured over a 24 h period in the

whole homogenate prepared from cultured rat liver slices from either phenobarbital-induced or control animals (Figure 3.5). In cultured slices from induced animals, the activity fell dramatically such that by 8 h, the activity was approximately 50% of that in fresh slices and after 24 h, only 12.5% of the O-deethylase activity remained. The ECOD activity of control rat liver slices was seven to ten times less than the activity from induced slices. The pattern of loss of activity in the control slices, however, almost exactly paralleled the changes observed in the activity from induced slices. These results were very similar to those reported by others using isolated rat hepatocytes (Greenlee and Poland, 1978; Edwards *et al.*, 1984).

3.1.6 NPSH content of cultured liver slices

Following an initial decline, the NPSH content of slices remained approximately constant between 2 and 24 h (Figure 3.6). In cultured hepatocytes, an initial and notable decrease and then an increase to or above initial levels has been reported (Morrison *et al.*, 1985; Hayes *et al.*, 1986).

3.1.7 Detection of newly synthesised protein by SDS-PAGE and autoradiography

Figure 3.7 shows an autoradiograph of radiolabelled proteins from liver slices cultured for up to 24 h. SDS-PAGE profile of newly synthesised proteins showed an increase in the intensity of bands corresponding to 67 kD in slices and in medium, in which the newly synthesised proteins were secreted, with respect to incubation time.

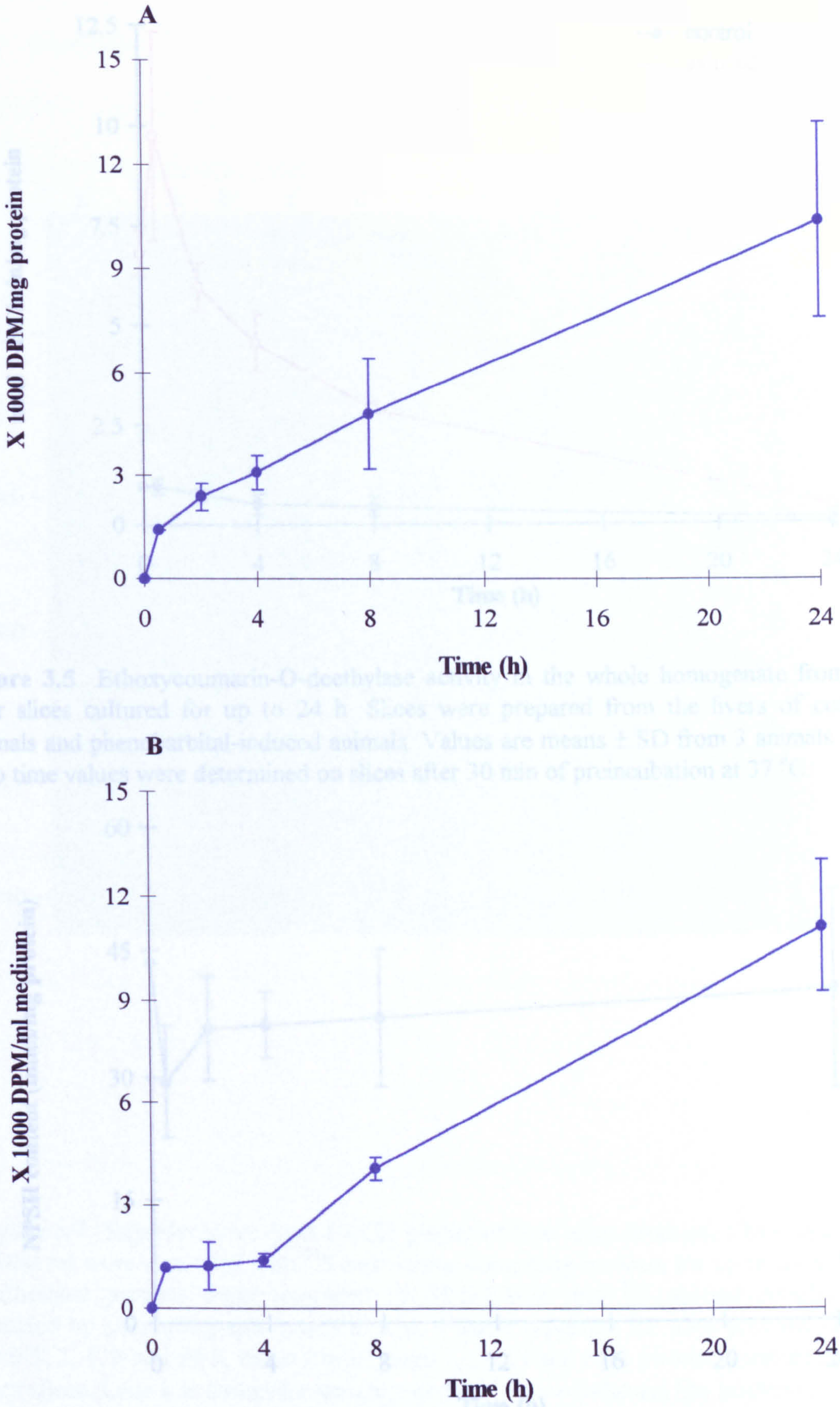


Figure 3.4 Protein synthesis and secretion from rat liver slices cultured for 24 h. Protein synthesis (A) was determined by the incorporation of [^{3}H] leucine into slice protein and protein secretion (B) by incorporation of [^{3}H] leucine into medium protein. Values are means ± SD from at least 3 animals.

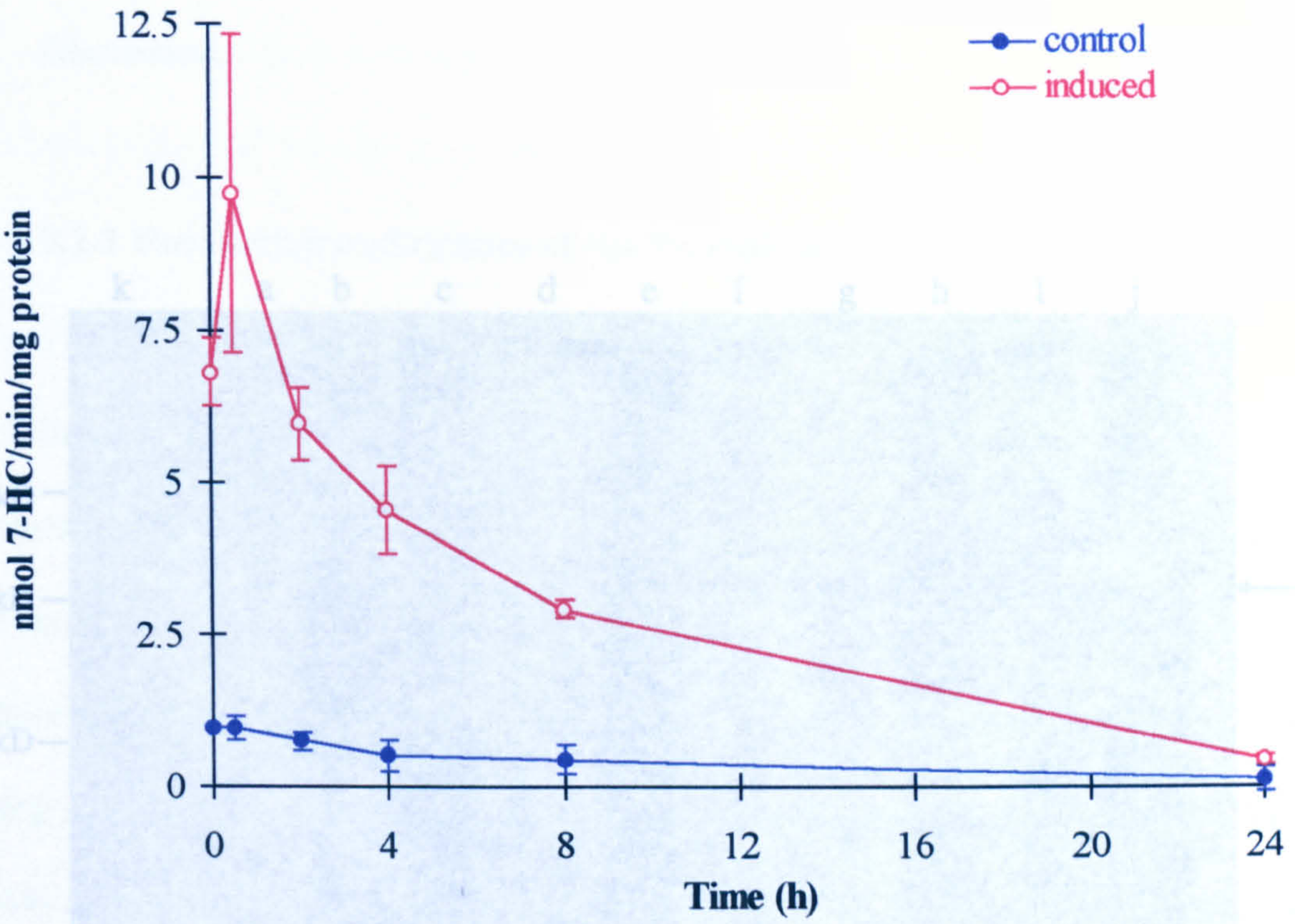


Figure 3.5 Ethoxycoumarin-O-deethylase activity in the whole homogenate from rat liver slices cultured for up to 24 h. Slices were prepared from the livers of control animals and phenobarbital-induced animals. Values are means ± SD from 3 animals. The zero time values were determined on slices after 30 min of preincubation at 37 °C.

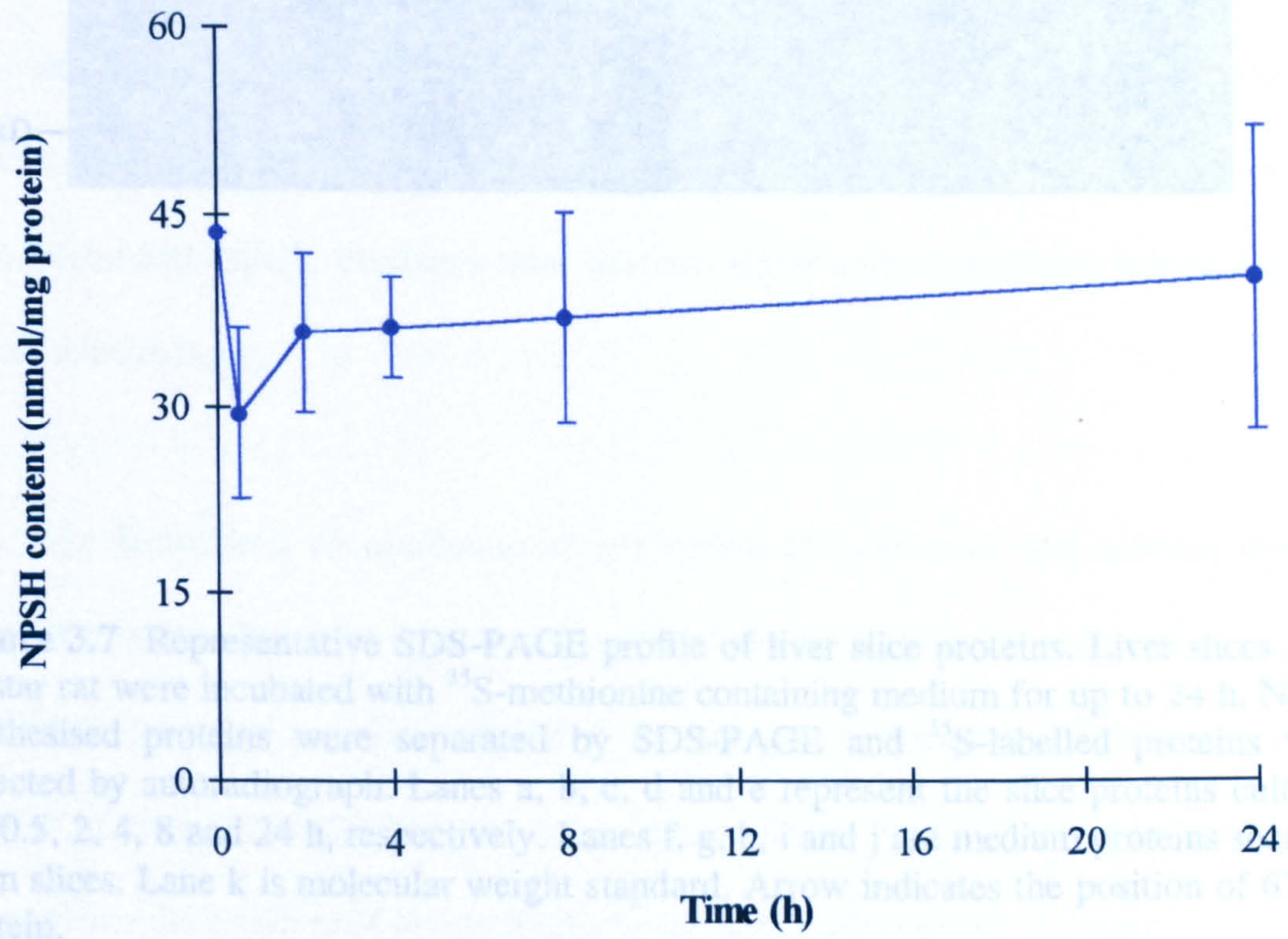


Figure 3.6 NPSH content of rat liver slices over 24 h of incubation. Values are means ± SD from 3 animals. The zero time value was determined on slices after 30 min of preincubation at 37 °C.

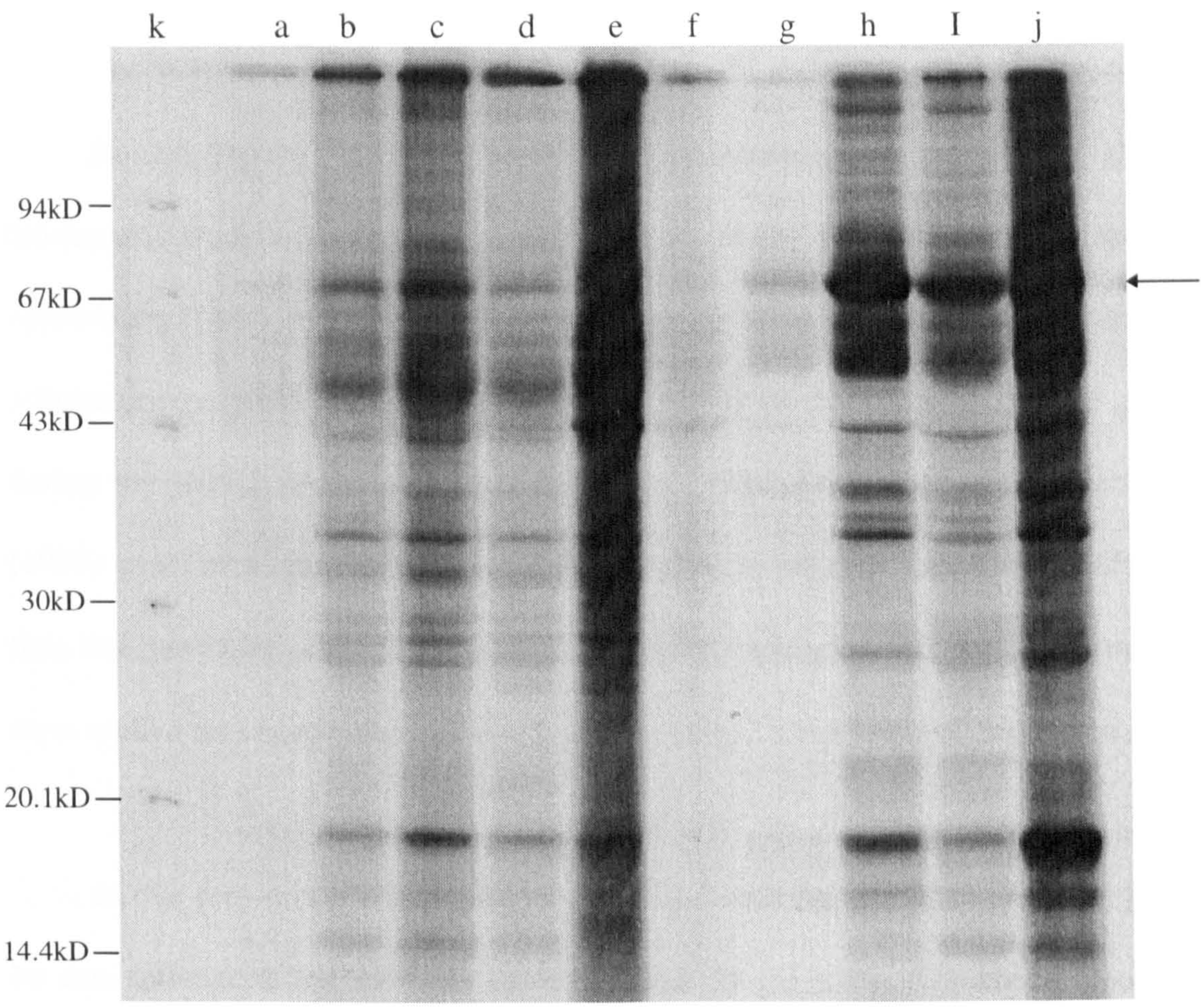


Figure 3.7 Representative SDS-PAGE profile of liver slice proteins. Liver slices from Wistar rat were incubated with ^{35}S-methionine containing medium for up to 24 h. Newly synthesised proteins were separated by SDS-PAGE and ^{35}S-labelled proteins were detected by autoradiograph. Lanes a, b, c, d and e represent the slice proteins cultured for 0.5, 2, 4, 8 and 24 h, respectively. Lanes f, g, h, i and j are medium proteins secreted from slices. Lane k is molecular weight standard. Arrow indicates the position of 67 kD protein.

3.2 Discussion

3.2.1 Production and culture of rat liver slices

Several reports have addressed the disadvantages of using liver slices for biochemical studies (Krebs *et al.*, 1974; Ichihara *et al.*, 1982) or for the evaluation of cytotoxicity (Grisham *et al.*, 1978). The major problem is spontaneous occurrence of cellular degeneration. This cellular degeneration may be due to tissue damage produced during the slicing process (Pollard & Dutton, 1982; Sturdee *et al.*, 1983). Trowell (1959) concluded that the trauma produced when tissue was sliced was much greater than that produced when tissue cubes or spheres were used. He postulated that slices were useless for organ culture although adequate for 2 h biochemical experiments.

In this project the disadvantages associated with the use of tissue slices prepared by conventional slicing methods were bypassed by using the Krumdieck tissue slicer. This instrument rapidly produces thin, uniform slices under conditions which result in minimal tissue injury.

The Krumdieck slicer allows the production of more than 100 uniform, thin rat liver slices in less than 1 h under very favourable conditions. Because each slice could be used as an individual experimental unit, it was possible to design experiments in which a number of slices obtained from the same liver could be used. Thus, fewer animals are required and the problem of interindividual variation is eliminated.

A new incubation technique was devised that allows adequate nutrient and gas exchange necessary for the survival of slices during incubation. Until now, the most frequently used incubation system for liver slice studies has been the dynamic organ culture system. This system rotates a slice adhering to a stainless-steel mesh through the incubation medium and atmosphere of the vial in a cyclical manner. This technique has been used successfully, especially in studies of the toxic effects of volatile substances (Dale *et al.*, 1988; Ghantous *et al.*, 1990, 1991). However, for toxicity and metabolism studies, this system has the disadvantage that there is no permanent contact of the slice with the incubation medium in which the test substance is dissolved. Therefore, to provide a more viable *in vitro* preparation, a simple shaking incubation system was used. This system was based on the method of Connors *et al.* (1990) and modified by omission of the basket, teflon ring and stir ball from the well to minimise mechanical damage during shaking (Dogterom 1993). Carrying out the incubations in 24-well plates made changing the culture medium and removing slices a relatively easy operation.

The effect of shaking speed on tissue viability was investigated. Table 3.1 shows that the selection of 60 rpm shaking speed led to much better results. A higher speed (90 rpm) induced 3-fold greater release of LDH, ALT and PDE than that by the lower speed. The movement of slices during incubation might be a critical factor in keeping slices viable for a longer period, because inappropriate mixing of culture medium has negative influences on the oxygen supply and causes mechanical damage (Smith *et al.*, 1988).

Having established culture conditions for rat liver slices, it was necessary to characterise several sensitive indicators of slice viability. An extensive study of the

functional integrity of cultured adult rat liver slices was undertaken using a number of different biochemical viability indicators. While there are no "ideal" viability indicators and no single test supplies full information about the metabolic competence of cultured liver cells, the criteria to be used should at least question the integrity and function of the plasma membrane and the metabolic performance of the cells (Krebs *et al.,* 1979). The parameters of slice viability which were chosen, therefore, were directed at fulfilling these minimal requirements for functional integrity and providing an integrated picture of the maintenance of various biochemical functions in the cultured liver slices over a period of up to 24 h.

3.2.2 Indicators of membrane integrity

The plasma membrane is the first part of the cell to be exposed to toxins, and it is therefore not surprising that it is a common site of toxic damage. Determination of the leakage of cytoplasmic enzymes into the surrounding media has been used by many workers as an indicator of cytotoxicity. Intracellular K^+ levels have been found to be one of the most sensitive indicators of cellular injury in isolated hepatocytes relative to other viability parameters (Baur *et al.,* 1975). Thus, total protein, PDE, ALT and LDH leakage was determined to measure slice membrane integrity, and intracellular K^+ retention was determined to monitor not only cellular membrane integrity, but also cellular energy.

3.2.2.1 Leakage of total protein, PDE, ALT and LDH

The protein content of cultured tissue is a commonly used test to measure *in vitro* toxicity. Thus, slice protein content was employed as an indicator of hepatocyte viability. The leakage of slice protein was approximately 25% after 24 h of incubation. This finding is similar with the work of Beamand *et al.* (1993) who reported 25-30% release of protein content from rat liver slices cultured for 24 h. The average protein content in slices observed in our studies was approximately 50-75 mg/g wet weight. This is considerably lower than that determined by Glockner and Muller (1995), in which the protein content of rat liver slices made by a McIlwain Tissue Chopper with 500 µm thickness was approximately 250 mg/g wet weight.

PDE is a membrane bound enzyme and was chosen as an indicator of plasma membrane integrity. Less than 5% of slice PDE activity was released within 24 h. LDH and ALT were also used for monitoring the loss of plasma membrane integrity. These enzymes have a different cellular distribution to PDE with LDH being cytosolic whilst ALT is both cytosolic and mitochondrial. At 4 h, release of ALT was 5%, this compares well with the work of Tyson and Green (1987), where ALT release at 4 h from hepatocyte suspensions was 6.2%. After 24 h, ALT release was approximately 20% which is in agreement with previous work on liver slices by Fisher *et al.* (1995b) who observed 25% release of ALT over 24 h incubation.

Baur *et al.* (1975) measured the release of lactate dehydrogenase (LDH) from hepatocytes in primary culture and found a highly positive correlation with trypan blue

permeability. The leakage of LDH is comparable to that reported in liver slices cultured in Waymouth's/Hepes medium (Fisher *et al.*, 1995a). LDH and ALT release is 10- and 3-fold that of PDE, respectively. It is likely, therefore, that membrane fragmentation is necessary for PDE release whilst release of LDH and ALT occurs through a 'leaky' plasma membrane. Of the three enzymes, LDH was the most sensitive indicator presumably because this enzyme is exclusively cytosolic, whilst ALT is located in both the cytosol and mitochondrial matrix (Agius and Tosh, 1990), and it is to be expected that the mitochondrial enzyme would only be released in more extreme toxicity.

3.2.2.2 K^+ content

Alterations in intracellular ion concentrations are indicative of cellular injury and/or death. In slices, where multiple cell types are present and stratification is maintained, changes in ion concentrations are used as a general index of viability. The actual site of the lesion cannot be elucidated using this parameter alone. However general, such indices are essential in determining time courses of toxicity and concentration-response relationship of a toxin in slices. The permeability of cells to certain ions that are normally concentrated either intracellularly or extracellularly is a reflection of cellular injury. Influx of Na^+ and Ca^+ and efflux of K^+ are indicative of severe damage to intramembranous ion pumps (Grisham, 1979). Baur *et al.* (1975) found the intracellular levels of Na^+ and K^+ to be most sensitive indicators of hepatic damage when compared with various other parameters of cell viability in freshly isolated cells. Others have further suggested that the K^+ content of cells, in addition to being a sensitive index of cell membrane integrity, is also very useful as a monitor for chemically-induced responses (Medzihradsky and

Marks, 1975). Thus, the leakage of intracellular K^+ is a sensitive parameter of cell membrane integrity which can be used in addition to the enzyme leakage tests (Ramos *et al.*, 1983; Santone and Acosta, 1984). Because K^+ ions are much smaller than the enzymes molecules released from injured cells, K^+ leakage may provide more subtle evidence of membrane damage by xenobiotics (Acosta *et al.*, 1987). Klaassen and co-workers have shown that K^+ leakage is a sensitive measure of the toxicity of heavy metals to isolated rat hepatocytes (Stacey *et al.*, 1980; Stacey and Klaassen, 1981). However, the results presented in this study showed that leakage of LDH is a more sensitive parameter than loss of K^+.

In this study, the concentration of K^+ in precision-cut rat liver slices was found to be approximately 1000 nmol/mg protein (equivalent to 60 µmol/g wet weight). This finding is consistent with the work of Smith *et al.* (1988) who reported the K^+ content of adult rat liver slices was 70-80 µmol of K^+/g wet weight in rat liver slices. However, different values for K^+ content in cultured rat liver slices have been reported by others. For example, Martin and McLean (1995) reported 400-500 nmol of K^+/mg protein whilst Wolfgang *et al.* (1991) observed K^+ values as low as 130 nmol/mg protein. Several other authors have reported K^+ values ranging from 15 to 100 µmol/g wet weight (Connors *et al.*, 1996; Dogterom, 1993; Baumann *et al.*, 1996; Sidelmann *et al.*, 1996; Muller *et al.*, 1998).

Liver slices cultured in the simple shaking incubation system were found to maintain their K^+ content with losses of only 10-20% after 24 h in culture. This finding is in contrast with the work of Campbell and Hales (1971) in which the K^+ content fell

steadily in static cultures of adult rat liver slices, reaching 50-60% of normal by 6 h. The time course of slice K^+ content is shown in Figure 3.3A and is very similar compared with that reported by Smith *et al.* (1988) using dynamic organ culture system and by Dogterom (1993) using 12-well culture plates. A relatively low concentration of K^+ was measured immediately after slicing and at least 2 h of incubation was necessary to restore the K^+ content to 1000-1100 nmol/mg protein. Similarly, Olinga *et al.* (1997) found that the K^+ content of cultured liver slices in several different incubation systems was restored by 1.5 h of preincubation. The K^+ content of liver slices in this study was 4-fold higher than the value reported by Wolfgang *et al.* (1990a) who also found that the initial low slice K^+ content under cold conditions was reversed over a period of approximately 1 h after incubation at 37 °C. The observation of an initial low content and subsequent recovery may be explained as a temperature-dependent reequilibration phenomenon that occurs upon warming the slices as they are transferred from ice-cold buffer (used for the preparation of slices) to warmer conditions of incubation. Barnabei *et al.* (1974) also observed a 44% decrease in the intracellular K^+ content of liver cells following incubation at 4 °C, but demonstrated a similar recovery to a level greater than the initial K^+ content during a successive incubation at 37 °C. In other studies employing liver slices from adult rats, Van Rossum and Russo (1981) demonstrated a two-fold increase in the K^+ content of liver slices following the transfer of these slices from 1 °C to 38 °C. Smith *et al.* (1988) reported approximately 2-fold increase in the K^+ content of liver slices following the transfer of slices from 4° C to 37 °C. There was a two-fold increase in the K^+ content following the transfer of liver slices from 4° C to 37 °C reported by Wolfgang *et al.* (1991). The time for this recovery was approximately 1-1.5 h, which is in good agreement with observations made in this study.

These results suggest that cultured liver slices in a simple shaking incubation system are better able to re-establish and maintain their intracellular levels of K^+ than hepatocytes isolated by collagenase perfusion. Cells isolated by this method have been shown to contain significantly reduced levels of K^+ (up to a 60% loss of the total K^+ content) upon isolation and fail to re-establish normal levels during incubation (Barnabei *et al.*, 1974). Quistorff *et al.* (1973) have suggested that the initial loss of intracellular K^+ may be due to a combination of factors including the low temperature during the cell wash and the high flow rate and pressure applied in the isolation procedure. The membrane damage possibly incurred by the cells during the collagenase perfusion step could lead to a leak of this intracellular ion (Tanaka *et al.*, 1978).

3.2.3 Cell functional markers

Biochemical assays based on vital functions of cells are usually easier to quantify, are more objective, and are readily automated. In addition, these may occur long before cell death. ATP content, protein synthesis and secretion and P-450 activity were chosen to indicate the cellular functions of cultured liver slices.

3.2.3.1 ATP content

ATP can be used as an indicator of cytotoxicity since it is the primary energy source at the cellular level. In order for the cell to function optimally it must maintain an intricate balance between energy production and consumption. The level of cellular ATP has been advocated by a number of authors as a measure of metabolic competence (Baur

et al., 1975; Krebs *et al.*, 1979; Page *et al.*, 1992), a claim supported by the demonstration that maintenance of ATP levels is a good prognostic indicator of success of hepatic transplantation (Sorrentino *et al.*, 1991). Baur *et al.* (1975) found that the intracellular levels of ATP and ADP in isolated hepatocytes declined at a rate that correlated with the rate of increase in the proportion of cells stained by trypan blue. Cellular ATP is commonly monitored in cytotoxicity studies with isolated hepatocytes and is dependent on the functional integrity of mitochondria (Tyson and Green, 1987). Although it is not as sensitive an indicator of cell damage as the K^+ content (Baur *et al.*, 1975), levels of ATP are a fairly sensitive indicator of cellular integrity (Klaassen and Stacey, 1982) and may be applied as an estimate of the functional metabolic capacity of liver cells (Dickson and Pogson, 1977). Following a recovery period of approximately 2 h, ATP content in cultured slices was maintained for 24 h. Maximal ATP content corresponded to approximately 75% of the reported normal levels determined in freeze clamped rat liver (Krebs *et al.*, 1974).

The initial decline in ATP content observed in these studies has been reported by others. Krebs *et al.* (1974), for example, showed that freshly prepared rat liver slices lost up to 70% of their total ATP and attributed this dramatic decline to the process of making slices. The actual ATP contents reported by Krebs *et al.* (1974) within less than a minute of making a slice, were, however, only 60% of the levels demonstrated in this thesis and this may reflect the more favourable conditions under which slices were prepared here. Similar results were obtained by Smith *et al.* (1986) who observed a nearly 1.5-fold increase in ATP content in liver slices cultured in dynamic organ culture system following their transfer from 4 °C to 37 °C. Dogterom (1993) also reported that

the ATP content of rat liver slices maintained in 12-well culture plates declined from 4 nmol/mg protein immediately after slicing to 3 nmol/mg protein, then recovered to 6 nmol/mg protein after 2 h and were then maintained. A similar recovery phase was also determined by Sidelmann *et al.* (1996) who observed a low level of ATP in slices (5 nmol/mg protein) immediately after slicing and a maximum of 10 nmol/mg protein reached after 2 h. Others found that an initial low level of ATP content in rat liver slices reached maximal levels after 12 h of incubation and remained high for the rest of the 24 h incubation period (Miller *et al.*, 1993). Singh *et al.* (1996) reported that the ATP content in trout liver slices was rapidly increased by approximately 40% during the first hour of incubation. All these observations can be explained by the utilisation of ATP exceeding its synthesis as slices are cooled, resulting in a lower steady ATP level. Since metabolism is totally arrested at 4 °C, ATP is neither synthesised nor utilised at this temperature (Russo *et al.*, 1977). Upon rewarming, the higher physiological steady state is re-established.

3.2.3.2 Protein synthesis and secretion

The ability of cultured cells to synthesise macromolecules, such as proteins, may be used as end-points for toxicity assays (Grisham, 1979). For tests of metabolic adequacy, quantitative measurements of the rates of biosynthesis provided a good indicator of functional competence in rat liver slices cultured for the 24 h. The active incorporation of radiolabelled leucine into proteins during the incubation also suggests that general metabolic deterioration is not occurring (Liberti *et al.*,1971). The incorporation of ^{3}H-leucine into TCA-precipitable slice proteins was nearly linear over 24 h incubation period

(Figure 3.4B). Similar results were obtained by Dickson and Pogson (1977) in cultured rat hepatocytes, by Smith *et al.* (1988) in rat liver slices and by Fisher *et al.* (1991c) in human liver slices. Using a static organ culture system of thin adult rat liver slices , Hart *et al.* (1983) demonstrated a linear incorporation of ^{3}H-leucine over 24 h. However, they also observed by light microscopy that 1/3 of the cultured tissue had deteriorated after 24 h which is in apparent contradiction with the observation that protein synthesis was maintained.

Another process which provides one of the best biochemical markers of hepatocyte activity is the secretion of radiolabelled proteins into the culture medium. This process changes rapidly and sensitively in response to changes in internal and external conditions (Ichihara *et al.,* 1982). Protein secretion is a multi-step, energy-dependent process which requires the maintenance of a variety of interactive cell functions and an intact system of microtubules (Margulis, 1973). Following a short lag period, the secretion of newly-synthesised protein into the culture medium was linear for up to 24 h. This lag period (2-4 h) corresponds with observations made in freshly isolated hepatocytes (Tanaka *et al.*, 1978), in incubated liver slices (Redman *et al.*, 1975) and in dynamically-cultured rat liver slices (Smith *et al.*, 1988). The latter cultures demonstrated levelling-off of protein secretion after 16 h, which could possibly be explained by depletion of amino acids in the medium (Smith *et al.*, 1988). This phenomenon was not apparent in the experiments reported here. Ichihara *et al.* (1982) have shown that a rapid decrease in the secretion of serum proteins from isolated hepatocytes occurs in amino acid-deficient medium and that this secretion could be restored by supplementing the medium with amino acids. Some radiolabelled protein in the culture medium may be released from damaged cells in

cultured slices. Retention or maintenance of both processes in slices following intoxication implies that transport of amino acids, synthesis of protein from amino acids and secretion of synthesised protein remains intact and functional.

3.2.3.3 P-450 activity

The activity of the hepatic microsomal drug-metabolising or cytochrome P-450 linked monooxygenases system frequently parallels the toxicity of many natural and synthetic chemicals (Paine and Legg, 1978; Paine, 1990). The maintenance of P-450 activity is of paramount importance for *in vitro* toxicity studies since many potentially toxic agents require metabolic activation for their toxic potential to be realised (Sipes and Gandolfi, 1982). For these reasons, an evaluation was made of the drug metabolising capacity in control and phenobarbital-induced rat liver slices during culture in the simple shaking culture system. ECOD activity has been used as a sensitive and convenient measure of the drug-metabolising activity of rat hepatocytes to monitor phenobarbital-induced isozyme of cytochrome P-450. 7-EC is a very interesting substrate which is oxidised by different species of cytochrome P-450 isozymes (Rogiers *et al.*, 1986). The O-deethylation of 7-EC results in the production of 7-HC which is readily estimated fluorimetrically. Over 8-24 h of culture period, the ECOD activity in either induced or control slices decreased more slowly (Figure 3.5), thus specific isozymes might have been lost. The activity in induced slices was between 7 to 10 times higher than that of controls. This finding was very similar to that reported by Greenlee and Poland (1978) using mouse liver, where O-deethylating activity was induced 7 to 10-fold by phenobarbital and by Smith *et al.* (1988) using rat liver slices, where ECOD activity in

control slices was 5 times less than the activity present in the phenobarbital-induced slices. The values which were obtained for the activity of this enzyme in fresh-isolated slices were very similar to those reported by others using liver slices (Sturdee *et al.*, 1983; Smith *et al.*, 1988) and isolated hepatocytes in suspension (Liu *et al.*, 1995).

The fairly rapid decline of the mixed-function oxygenase system in cultured liver slices from phenobarbital-induced animals was similar to those observed in hepatocyte culture, which lose more than 50% of their cytochrome P 450 activity during the first 24 h of incubation (Paine and Legg, 1978; Paine and Hockin, 1980; Maslansky and Williams, 1982; Paine, 1990) and in rat liver slices cultured in dynamic organ culture system, where only 50% of the ECOD activity remained after 20 h (Smith *et al.*, 1988). A significant decrease of ECOD activity in rat liver slices by a 24 h incubation was also confirmed by Glockner and Muller (1995).

3.2.4 Non-specific indicator: NPSH content

Hepatocytes located in the periportal zone of the acinus have a high GSH content relative to centrilobular hepatocytes (Jungermann and Kats, 1989). GSH is a tripeptide found within most mammalian cells which plays an important role in hepatic drug detoxification reactions effected by glutathione S-transferases (Reed and Orrenius, 1977). In the process of biotransformation of toxins, a number of highly reactive electrophilic compounds are formed. Some of these compounds can react with cellular constituents and cause cell death or induce tumour formation. The physiological role of GSH is to react with these electrophilic compounds and thus prevent their harmful

effects on the cells. However, exposure to very large amounts of such reactive substances can deplete the GSH, thereby resulting in marked toxic effects (Lu, 1985). Also, reactive metabolites can oxidise and thereby reduce the cellular GSH levels. Thus, if a cell is depleted of GSH, it is more vulnerable to toxic compounds and more and more evidence accumulates proving that GSH plays a fundamental role in cellular metabolism. As the most abundant intracellular sulphydryl, GSH is a key factor in the detoxification of electrophilic metabolites of xenobiotics. As the cosubstrate for GSH peroxidase, GSH prevents peroxidation of membrane lipids and, together with other antioxidants, plays an important role in maintaining cellular integrity (Flohe *et al.*, 1976). As the determinant of the sulphydryl/disulphide ratio, GSH also modulates the activity of a number of enzymes (Kosower and Kosower, 1978).

GSH content was measured by determining NPSH in cultured liver slices because most NPSH is GSH (Jurima-Romet *et al.*, 1996). NPSH content declined to approximately 70% of that observed in freshly-isolated slices within 0.5 h of culture (Figure 3.6). This finding is similar to that reported by Morrison *et al.* (1985) in primary cultures of rat heptocytes where reduced GSH dropped to 50% of that in freshly-isolated cells within 1 h in culture. The NPSH content reported here agrees well with that reported in isolated hepatocytes (Reed & Orrenius, 1977; Mertens *et al.*, 1996). Hayes *et al.* (1986) also found that the GSH concentration in isolated hepatocytes transiently decreased during the first 6 h in culture, then recovered and was maintained for 18 h.

3.2.5 Synthesis and secretion of specific proteins

Liver slices were incubated with medium containing ^{35}S-methionine for up to 24 h. Radiolabelled proteins were separated by SDS-PAGE and detected by autoradiography. The results demonstrated that cultured slices synthesised many new proteins some of which were then secreted into the cultured medium. This could be used as a practical sensitive index of hepatotoxicity and a potential means of studying the expression of stress proteins by various hepatotoxins.

In summary, it has been shown that liver slices can easily be incubated in shaking 24-well plates which maintains them viable and metabolically active for up to 24 h. As indicated by all parameters, functional integrity was satisfactorily maintained. It is possible, therefore, to study hepatotoxicity in this system and bromobenzene was chosen as a model hepatotoxin for further experimental work.

CHAPTER 4

INVESTIGATION OF BROMOBENZENE TOXICITY IN CULTURED RAT LIVER SLICES

4.1 Methods

The *in vitro* hepatotoxiciy of bromobenzene (BB) was studied in cultured rat liver slices over a period of 8 h. Animals were pretreated by intraperitoneal injection of either 0.9% saline or sodium phenobarbital as described in Section 2.2. Liver slices from control and phenobarbital-induced animals were prepared and cultured as described in Sections 2.3-2.6.

BB solutions were made up in DMSO and added to the incubation medium to the final BB concentrations required such that the final DMSO concentration in the culture medium was ≤ 1% (v/v). Control incubations contained 1% (v/v) DMSO alone. All slices were preincubated in DMEM for 30 min at 37 °C before any experiments were undertaken.

Cytotoxicity was evaluated between 0 - 8 h by measuring slice K^+ and ATP contents, protein synthesis and secretion, leakage of LDH, ALT and PDE, depletion of NPSH, and, histopathology. All indicators were determined and expressed as described in Section 2.8.

4.2 Results

4.2.1 Loss of K^+ and ATP from cultured slices by bromobenzene

The K^+ content of hepatocytes is believed to be a more sensitive indicator of the integrity of the plasma membrane than enzyme leakage (Baur *et al.*, 1975) and has been widely employed as an index of cell viability. Figure 4.1 shows that BB produced a dose- and time-dependent loss of intracellular K^+ and ATP over 8 h of incubation. Control slices maintained stable levels of K^+ and ATP for the duration of 8 h incubation. There were no differences in K^+ and ATP content between uninduced control, phenobarbital-induced and DMSO-treated slices. BB concentrations of 0.1 mM and above produced a significant losses of intracellular K^+ such that after 8 h incubation with 1 mM BB, slice K^+ content decreased to 20% of that in control slices (Figure 4.1A). Similar results were observed for the depletion of ATP content in slices exposed to BB (Figure 4.1B).

4.2.2 Leakage of LDH, ALT and PDE from cultured slices by bromobenzene

As shown in Figure 4.2, no differences were found in enzyme release between uninduced control, phenobarbital-induced control and DMSO-treated slices.

There was a relatively low level of leakage of LDH, ALT and PDE from cultured liver slices not exposed to toxin. This was most marked for LDH with approximately 25% release after 8 h incubation whilst only 10% of ALT and 2% of PDE was released over a similar time period (Figure 4.2). As shown in Figure 4.2, differences in enzyme

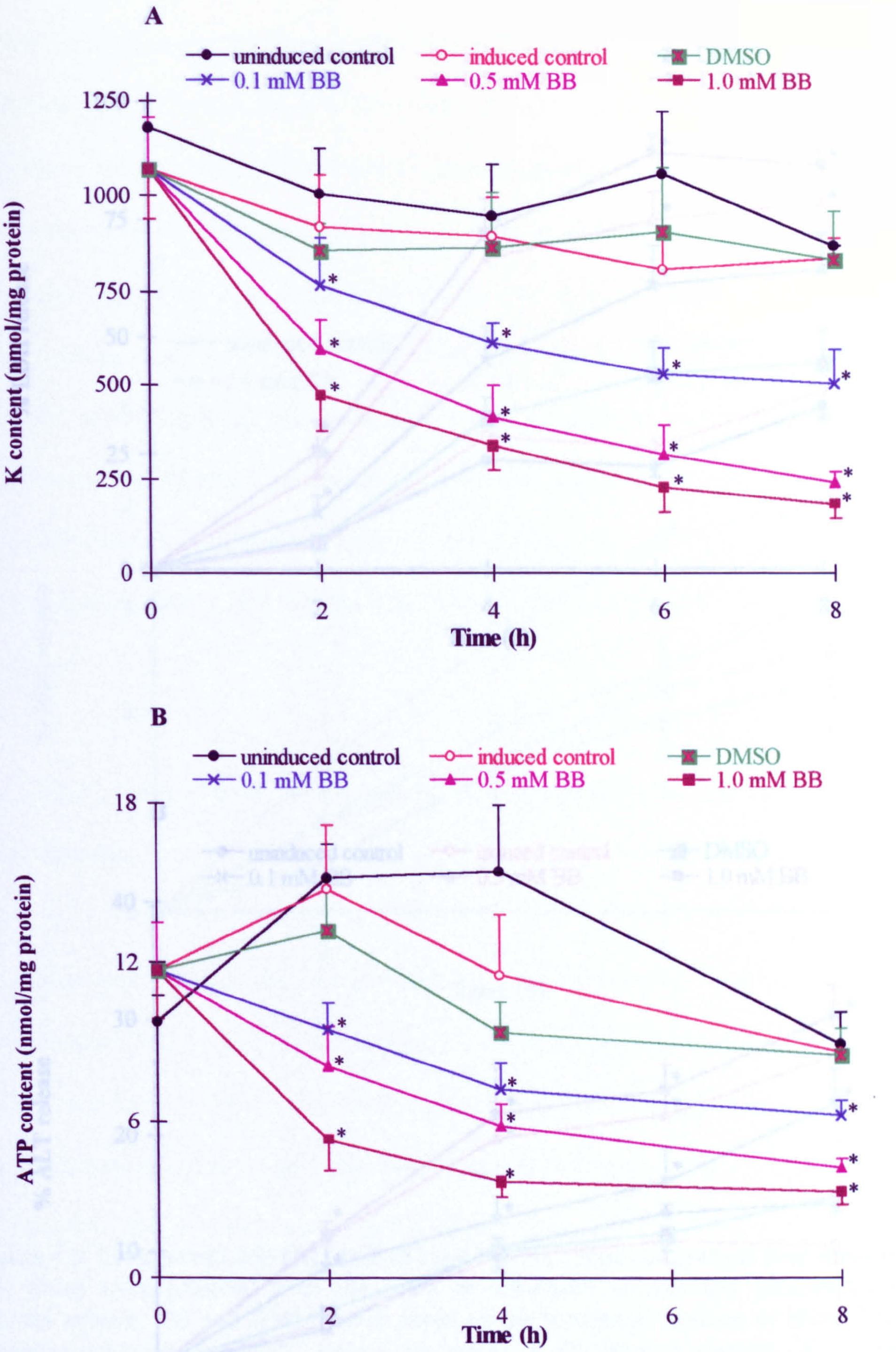
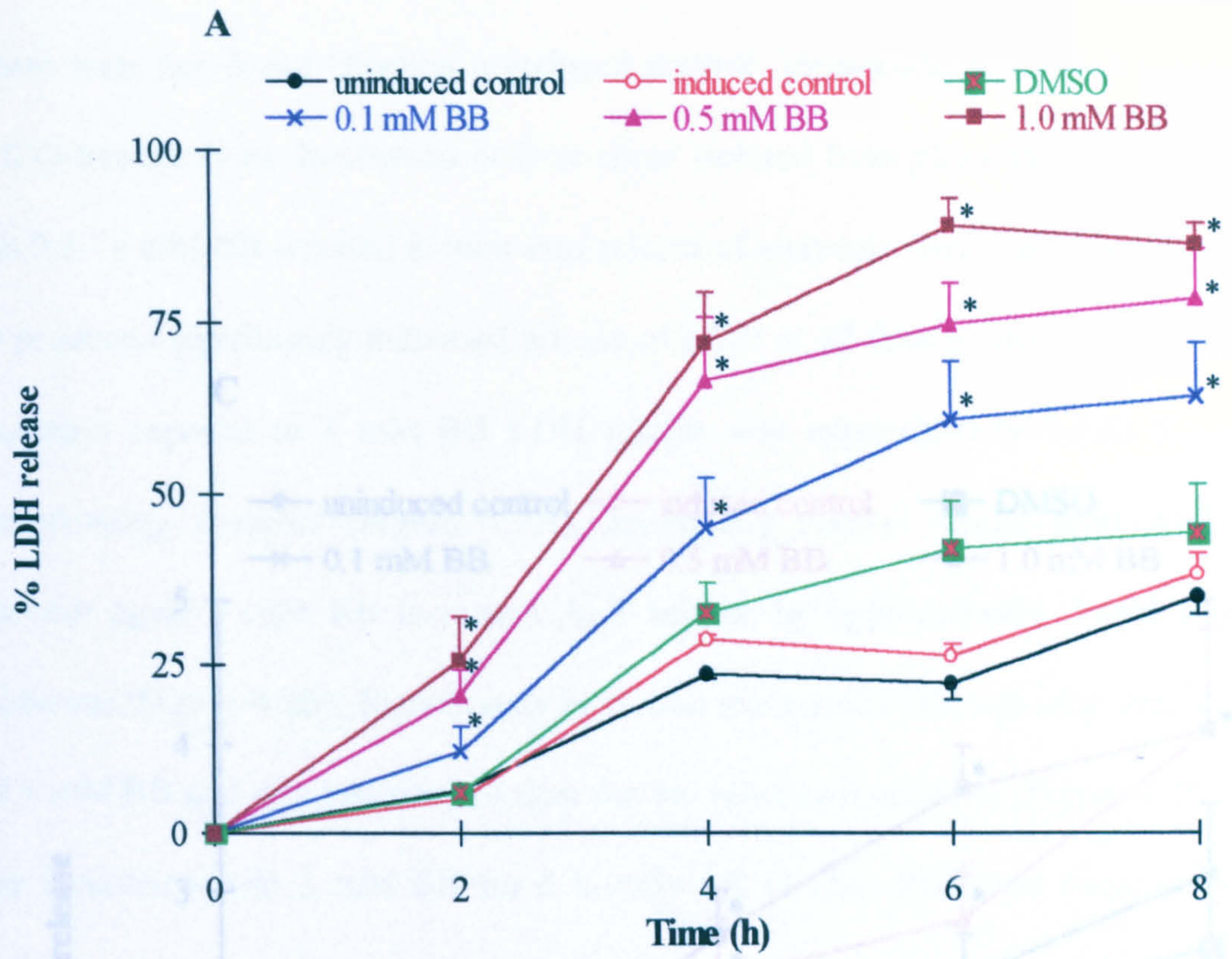


Figure 4.1 Loss of intracellular K^+ (A) and ATP (B) from cultured rat liver slices by BB. Slices were prepared from the livers of uninduced animals and phenobarbital-induced animals. BB and DMSO were added to the incubation medium of slices from phenobarbital-induced animals. Values are means ± SD from 4 animals. $^*P < 0.05$ compared to induced control.



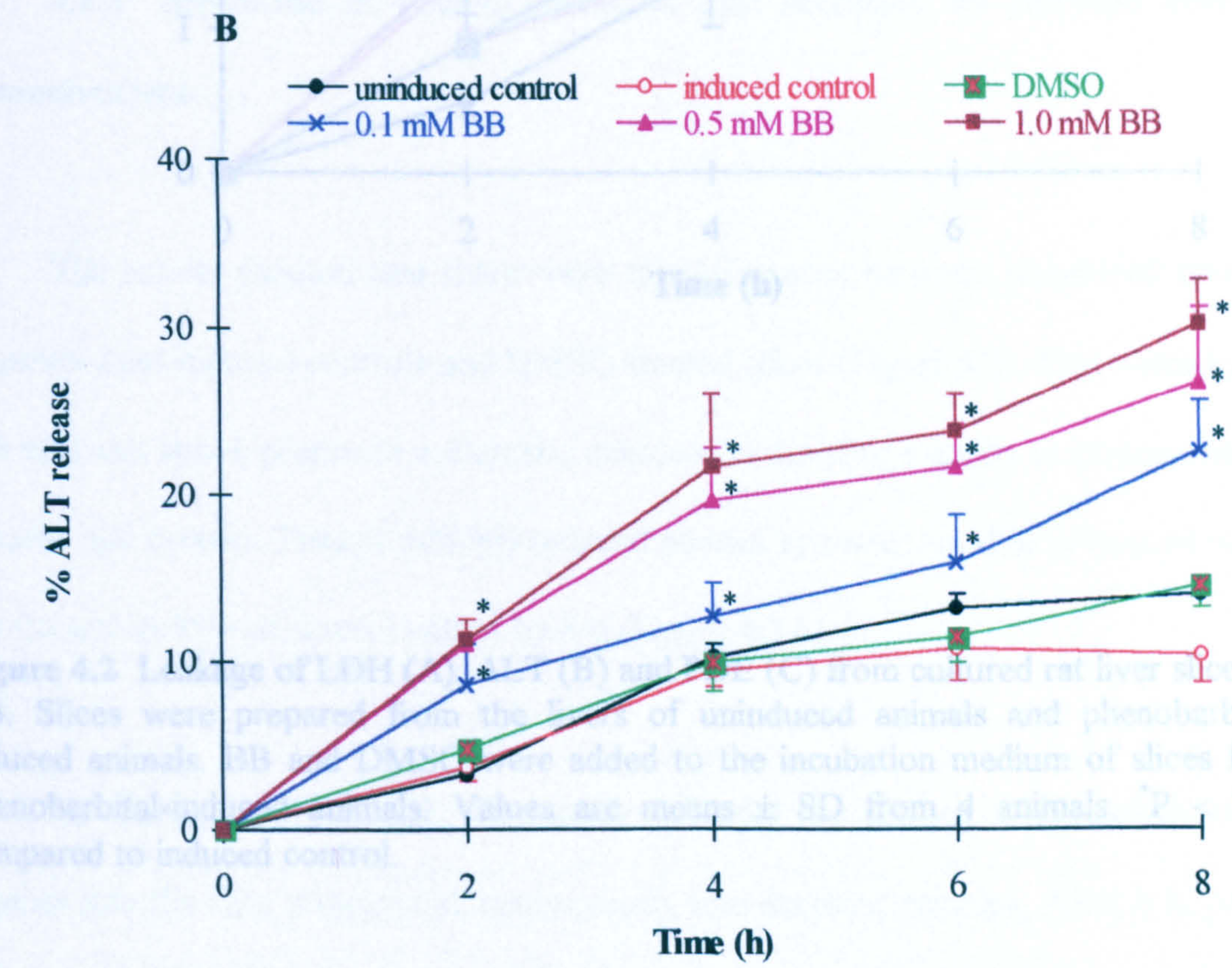


Figure 4.2 See over for legend.

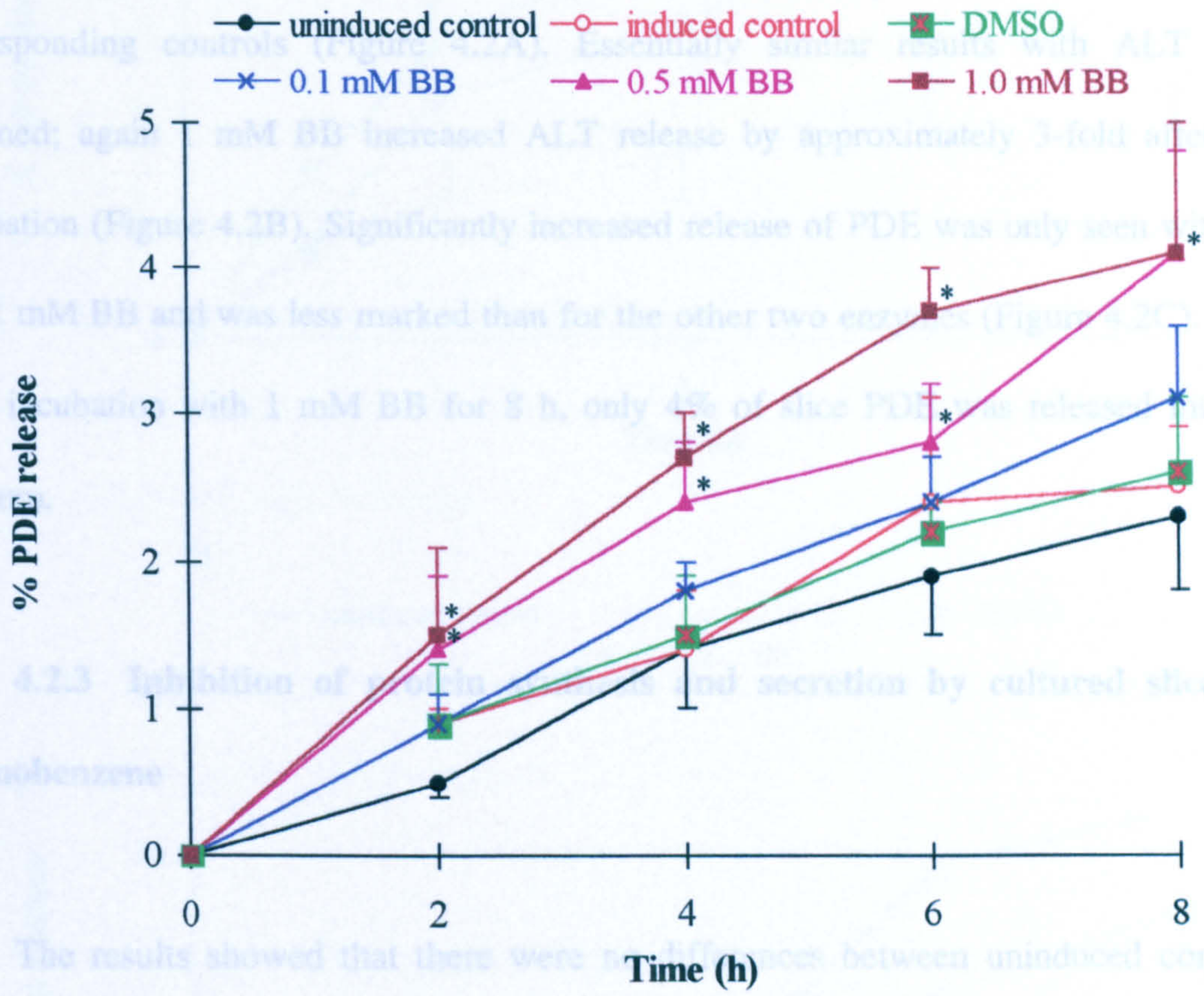


Figure 4.2 Leakage of LDH (A), ALT (B) and PDE (C) from cultured rat liver slices by BB. Slices were prepared from the livers of uninduced animals and phenobarbital-induced animals. BB and DMSO were added to the incubation medium of slices from phenobarbital-induced animals. Values are means ± SD from 4 animals. $^{*}P < 0.05$ compared to induced control.

release were not found between uninduced control, phenobarbital-induced control and DMSO-treated slices. Incubation of liver slices isolated from phenobarbital-induced rats with 0.1 - 1 mM BB resulted in increased release of enzymes. Thus, all concentrations of BB produced significantly increased release of LDH at all time points; indeed, after 8 h incubation exposed to 1 mM BB LDH release was approximately 3-fold that in the corresponding controls (Figure 4.2A). Essentially similar results with ALT were obtained; again 1 mM BB increased ALT release by approximately 3-fold after 8 h incubation (Figure 4.2B). Significantly increased release of PDE was only seen with 0.5 and 1 mM BB and was less marked than for the other two enzymes (Figure 4.2C). Even after incubation with 1 mM BB for 8 h, only 4% of slice PDE was released into the medium.

4.2.3 Inhibition of protein synthesis and secretion by cultured slices by bromobenzene

The results showed that there were no differences between uninduced controls, phenobarbital-induced controls and DMSO-treated slices (Figure 4.3). Concentrations of 0.5 mM and above produced a dramatic decrease in the slice's ability to incorporate ^{3}H-leucine into protein. Thus, 1 mM BB reduced protein synthesis to 55% of control values by 4 h and to 35% of control values by 8 h (Figure 4.3A).

As shown in Figure 4.3, BB rapidly inhibited the rate of incorporation of ^{3}H-leucine into the slice protein and, consequently into secreted proteins. After 8 h, protein

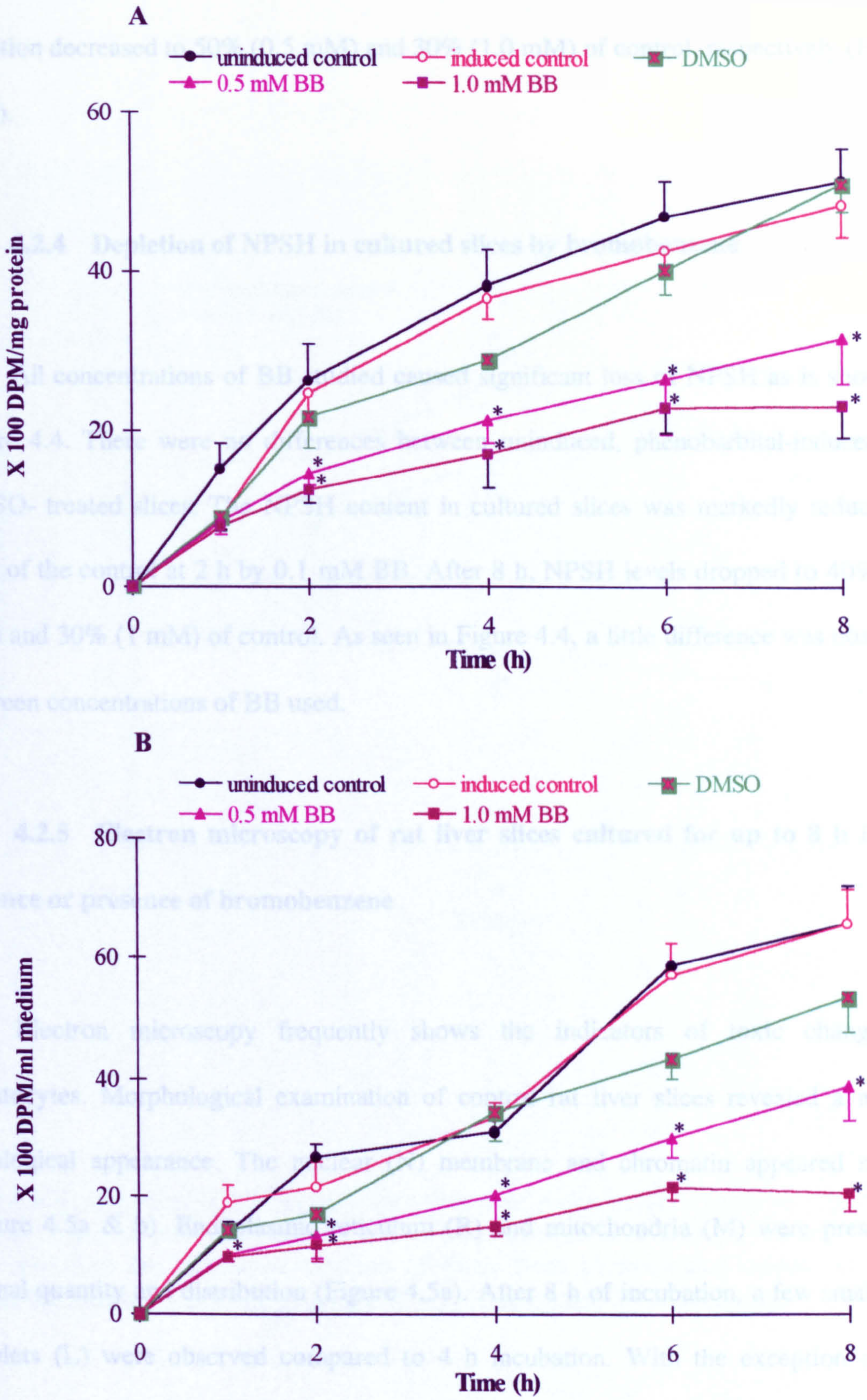


Figure 4.3 Inhibition of protein synthesis (A) and secretion (B) by cultured rat liver slices by BB. Slices were prepared from the livers of uninduced animals and phenobarbital-induced animals. BB and DMSO were added to the incubation medium of slices from phenobarbital-induced animals. Values are means ± SD from 4 animals. $^{*}P < 0.05$ compared to induced control.

secretion decreased to 50% (0.5 mM) and 30% (1.0 mM) of control, respectively (Figure 4.3B).

4.2.4 Depletion of NPSH in cultured slices by bromobenzene

All concentrations of BB studied caused significant loss of NPSH as is shown in Figure 4.4. There were no differences between uninduced, phenobarbital-induced and DMSO- treated slices. The NPSH content in cultured slices was markedly reduced to 50% of the control at 2 h by 0.1 mM BB. After 8 h, NPSH levels dropped to 40% (0.1 mM) and 30% (1 mM) of control. As seen in Figure 4.4, a little difference was observed between concentrations of BB used.

4.2.5 Electron microscopy of rat liver slices cultured for up to 8 h in the absence or presence of bromobenzene

Electron microscopy frequently shows the indicators of toxic changes in hepatocytes. Morphological examination of control rat liver slices revealed a normal histological appearance. The nuclear (N) membrane and chromatin appeared normal (Figure 4.5a & b). Endoplasmic reticulum (R) and mitochondria (M) were present in normal quantity and distribution (Figure 4.5a). After 8 h of incubation, a few small lipid droplets (L) were observed compared to 4 h incubation. With the exception of this change, cell organelles were well preserved. Mitochondria (M) exhibited little structural variation within a near normal range. Nuclei and rough endoplasmic reticulum (RER) were well maintained (Figure 4.5b).

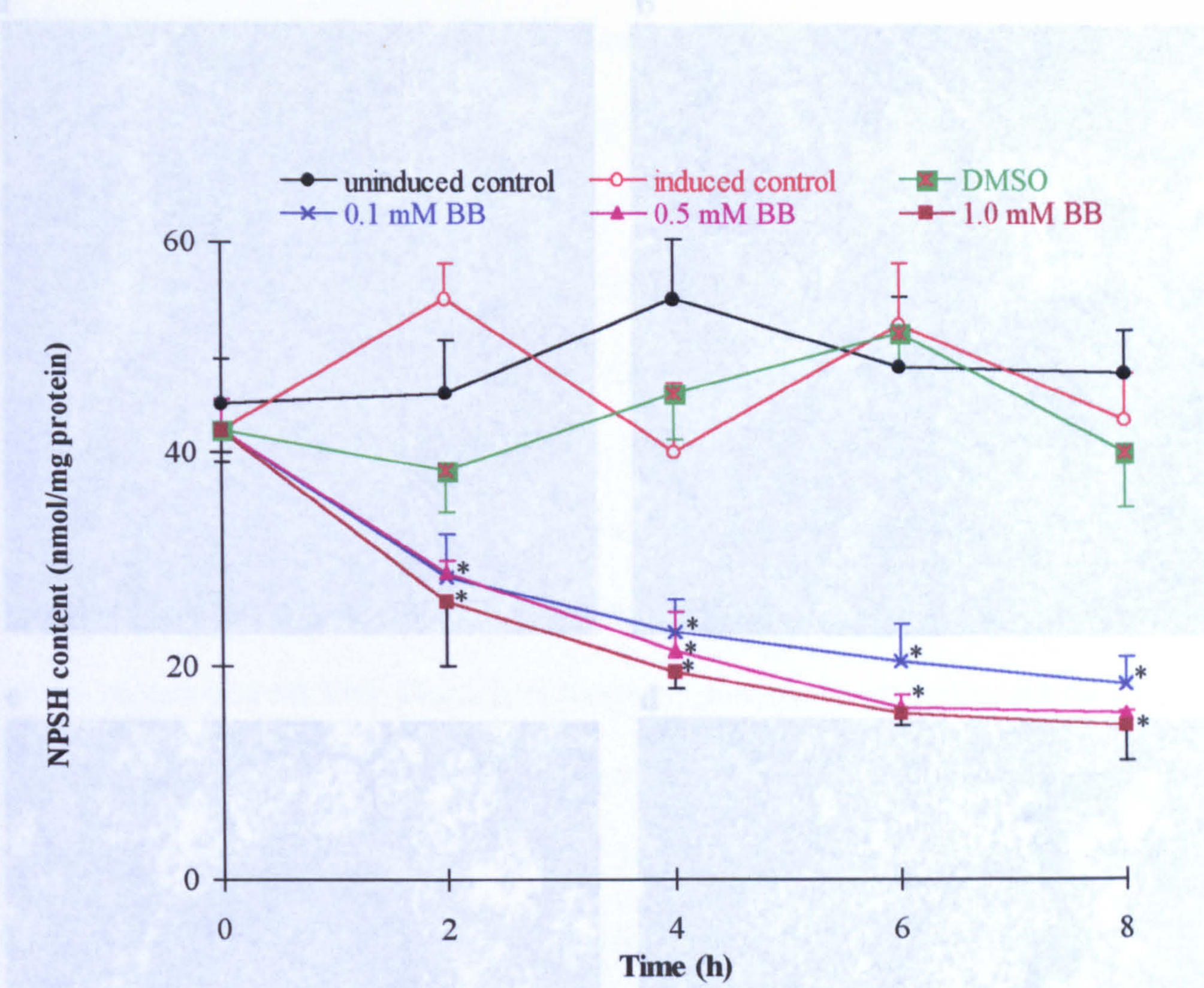


Figure 4.4 Depletion of NPSH in cultured rat liver slices by BB. Slices were prepared from the livers of uninduced animals and phenobarbital-induced animals. BB and DMSO were added to the incubation medium of slices from phenobarbital-induced animals. Values are means ± SD from 4 animals. $^{*}P < 0.05$ compared to induced control.

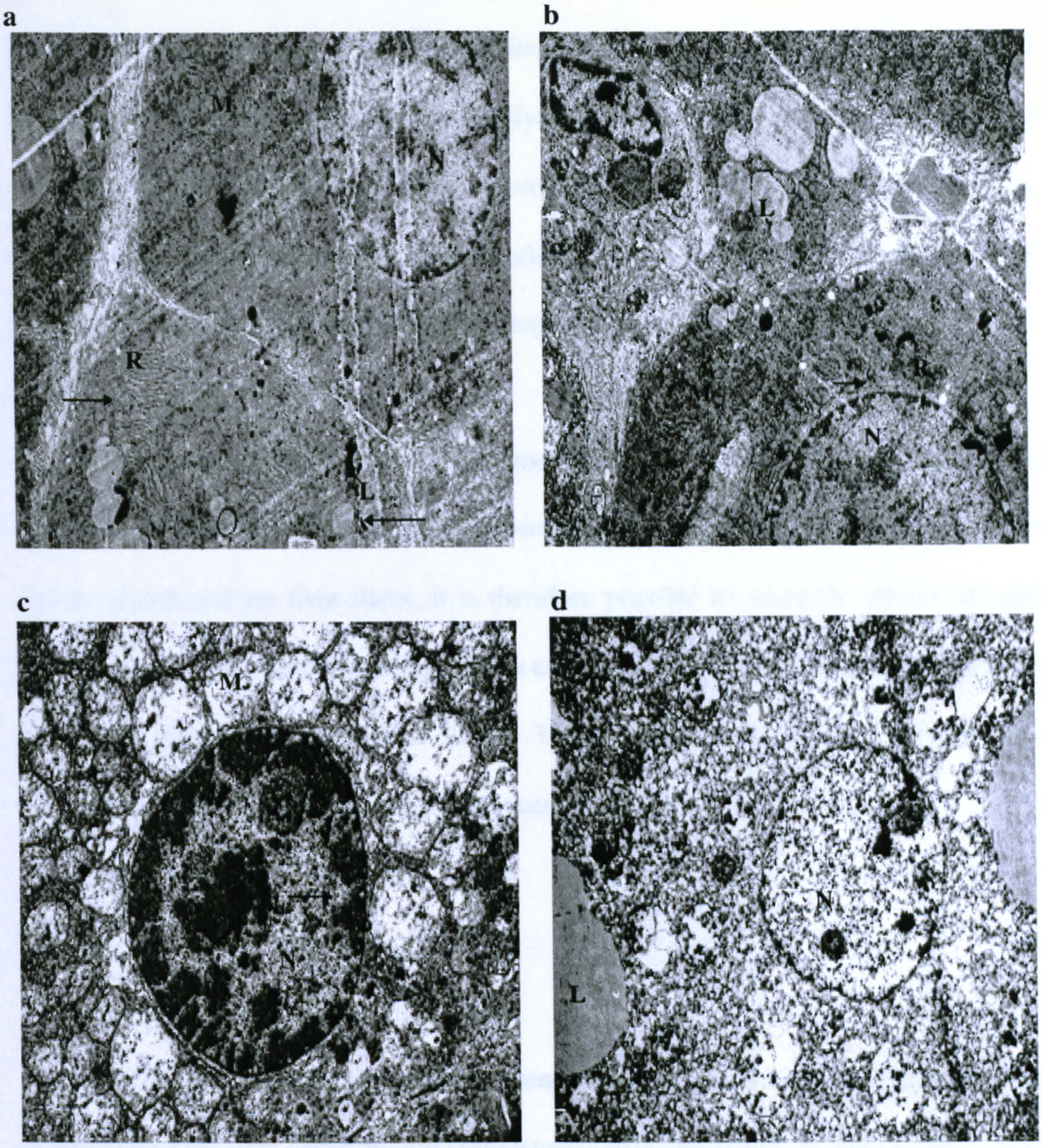


Figure 4.5 Electron micrographs of part of rat liver slices. Panels a and b show control slices cultured for 4 h and 8 h, respectively. Panels c and d show liver slices cultured for 4 h and 8 h, respectively, in the presence of 1 mM bromobenzene. N, nucleus; M, mitochondria; R, endoplasmic reticulum; L, lipid droplet.

Examination of the liver slices exposed to 1 mM BB under the electron microscopy showed that they were severely damaged. Gross disruption of cytoplasmic organelles had occurred (Figure 4.5c & d). After 4 h, mitochondria were swollen or even vacuolarly degenerated. RER was dilated and vesicularly transformed. The membrane of the nucleus showed a clumped chromatin pattern developing a hyperchromatosis (Figure 4.5c, arrow). More extensive damage occurred after 8 h of incubation with BB. Nuclei were now swollen and spherical and large lipid droplets were observed (Figure 4.5d).

In summary, a concentration- and time-dependent of toxicity of BB was clearly observed. The hepatotoxicity of BB has been well established and validated using the system of cultured rat liver slices, it is therefore possible to study the effects of aged garlic extract in this *in vitro* system. BB at a concentration of 1 mM induced a significant and acute injury to the rat liver slices. Thus, 1 mM was chosen as ideal toxic concentration of BB and used in the subsequent experiments.

4.3 Discussion

The classical hepatotoxin bromobenzene (BB) was chosen for studies of toxicity using the rat liver slice organ culture system previously developed. In these studies, slices were prepared from rats which had been induced by phenobarbital; induction of cytochrome P-450 2B1 and P-450 2B2 increases the hepatotoxicity of BB. Slice K^+ and ATP content, LDH, ALT and PDE leakage, protein synthesis and secretion, depletion of NPSH content and electron microscopy were used as indicators of chemically-induced cell injury.

BB at 1 mM produced a time-dependent loss of slice K^+ and ATP over a 8 h culture period resulting in a 80% and 60% loss of slice K^+ and ATP compared to control slices respectively (Figure 4.1). The same concentration of BB also produced liver injury as evidenced by significant increases in LDH, ALT and PDE leakage over 8 h of incubation (Figure 4.2). Other workers have demonstrated a requirement for phenobarbital induction in order to produce toxicity with BB *in vitro*. For example, Dankovic and Billings (1984) demonstrated that 1 mM BB results in a loss of LDH from hepatocytes isolated from phenobarbital-induced rats. In the work of Hayes *et al.* (1984), it was shown that hepatocytes from phenobarbital-induced animals exposed to 1.6 mM BB for 24 h released 70% of their total LDH activity. Thor and Orrenius (1980) demonstrated similar effects of BB using hepatocytes from phenobarbital-induced, diethyl maleate-treated rats. Diethyl maleate was used to deplete hepatic GSH and therefore increase further sensitivity to BB. In their system, increased BB cytotoxicity was also characterised by marked effects on plasma membrane structure and function and BB at 0.6 mM caused a 70% loss of intracellular K^+ after 3 h. Day *et al.* (1992) also reported that the centrilobular necrosis caused by BB can be exacerbated with the induction of P-450 2B by phenobarbital. Smith *et al.* (1985) demonstrated over a 6 h incubation period that BB was only toxic to rat liver slices when the animals were phenobarbital induced. Gottschall *et al.* (1984) reported that 1 mM BB resulted in 50% ALT release and 70% loss of K^+ from isolated hepatocytes. Smith *et al.* (1986) demonstrated that BB at 1 mM produced a two-fold increase in LDH activity of the media and a 70-80% loss of K^+ from liver slices prepared from phenobarbital-induced rats. These findings are in complete agreement with the results presented here.

Results obtained with rat liver slices showed a concentration-dependent relationship for BB toxicity from 0.1 to 1.0 mM. Concentrations of 0.1 mM or greater were cytotoxic after 4 h producing a 35% and 50% loss of slice K^+ and ATP respectively and after 6 h, concentrations of 0.5 mM or above produced a 3-fold increase in LDH release and a 2-fold increase in ALT release. After 8 h, 1 mM BB caused a 2-fold increase in PDE leakage. The findings were consistent with a report by Smith *et al.* (1986) in rat liver slices and by Duthie *et al.* (1994) who demonstrated that BB caused a concentration (0-10 mM) and time-dependent (0-3 h) decrease in human HepG2 cell viability.

The measurement of protein synthesis and secretion are another two parameters of BB toxicity which were studied in cultured rat liver slices. By 2 h, significant inhibition of ^{3}H-leucine incorporation into the slice proteins and newly-synthesised proteins secreted into the culture medium were observed (Figure 4.3). Again, there was little difference in the extent of this inhibition produced by either 0.5 or 1 mM BB. A similar level of inhibition was produced in either isolated hepatocytes from phenobarbital-induced rats exposed to 0.6 mM BB for 3 h or in perfused livers from rats pretreated with BB (1 ml/kg, i.p.) for 4 h (Davis *et al.*, 1973). Smith *et al.* (1986) reported a similar inhibition of protein synthesis in cultured liver slices prepared from phenobarbital-induced rats. Further support for these findings can be also found in the studies of Fisher *et al.* (1995 a & b).

In this study, the NPSH content (mainly GSH) during the exposure period was evaluated because GSH plays many important roles in maintaining cell function and

processing reactive metabolites into its conjugates (Jollow *et al.*, 1974; Meister and Anderson, 1983). GSH conjugation has been generally considered to reduce the toxic effect of active substances. GSH has been also shown to play a critical role in the detoxification of BB toxicity in rats (Duthie *et al.*, 1994). Concentrations of 0.1 mM or more produced significant depletion of NPSH after 2 h compared to that observed in control slices. After 8 h in culture, only 30% of control NPSH content was observed in slices exposed to 1 mM BB. Dankovic and Billings (1985) demonstrated that BB at 3 and 5 mM depleted GSH to 13 and 9%, respectively, of the concentration in control rat hepatocytes at 2 h. Grewal *et al.* (1996) found GSH depletion in isolated mouse hepatocytes caused by 3 mM BB was significant by 3 h and at 6 h, approximately 70% of GSH was depleted compared with control levels. GSH content in isolated rat hepatocytes exposed to 2.4 mM BB was markedly reduced to 40% of the control after 3 h (Wu *et al.*, 1996).

Many workers (e.g., Thor *et al.*, 1978) have suggested that GSH depletion results in the accumulation of reactive intermediates leading to the observed cytotoxicity. The dose-dependent depletion of the tripetide coincides with a decrease in the excretion of the mercapturic acid and an increase in covalent binding (Timbrell, 1991). Thus, GSH protects the hepatocyte against the reactive metabolite, bromobenzene 3, 4-oxide by conjugating with it chemically or enzymatically. After a hepatotoxic dose, however, there is sufficient of the reactive metabolite to deplete the available hepatic GSH. The reactive metabolite is therefore not detoxified by conjugation with GSH, and is able to react with cellular macromolecules such as proteins (Timbrell, 1991). BB has been shown to produce GSH depletion both *in vivo* (Jollow *et al.*, 1974; Casini *et al.*, 1985; Maellaro *et*

al., 1990) and *in vitro* (Thor *et al.*, 1978; Mennes *et al.*, 1991; Wu *et al.*, 1996). Similarly, in this study, BB significantly depleted the NPSH content of rat liver slices. A significant reduction of the NPSH content in rat liver slices 2 h after exposure to BB indicates that depletion of GSH is an early event in the pathogenesis of BB toxicity and is consistent with *in vitro* findings in hepatocytes from other species such as human, monkey and dog (Mennes *et al.*, 1994; Tarauchi and Hirata, 1994).

Electron microscopy frequently provides the earliest indications of toxic changes in hepatocytes, degranulation and vacuolation of the rough endoplasmic reticulum being a particularly useful indicator. It is also invaluable in detecting proliferation of smooth endoplasmic reticulum, peroxisomes and mitochondria although minor changes in these organelles should not, in themselves, be taken as indicators of hepatotoxicity (Hinton and Grasso, 1993). Electron microscopy has already been successfully used to characterise toxic effects in precision-cut renal slices (Wolfgang *et al.*, 1990b). In this study, electron microscopy confirmed that the cells from cultured liver slices exposed to bromobenzene (1 mM) were necrotic. Observations of BB toxicity in rat liver slices have demonstrated vacuolisation and swelling of mitochondria, blebbing of the cytoplasmic membrane, chromatin clumping and dilation of the endoplasmic reticulum. The histological damage observed appeared to parallel the biochemical changes described above. This ultrastructural investigation suggests that it could have been early signs of cell damage.

In summary, BB at 1 mM caused a significant loss of slice K^+ and ATP compared with fresh-isolated slices, a rapid increase of enzyme leakage, notable inhibition of protein synthesis and secretion, rapid depletion of NPSH content, and, marked

morphological changes in hepatocytes by only 2 h. These observations confirm that BB has acute toxic effects on rat liver slices. Moreover, these effects were time- and dose-dependent. Thus, 1 mM was chosen as the concentration of BB at which to assess any possible hepatoprotective effect of garlic and its constituents.

CHAPTER 5

PROTECTIVE EFFECTS OF AGED GARLIC EXTRACT AGAINST BROMOBENZENE TOXICITY *IN VITRO*

The reported ability of garlic preparations and related organosulphur compounds to protect against certain cytotoxicities has been discussed in Section 1.6. In this chapter, the ability of aged garlic extract (AGE) to modulate the hepatotoxicity of bromobenzene *in vitro* has been investigated.

5.1 Methods

Animals were pretreated by the intraperitoneal injection with sodium phenobarbital as described in Section 2.2. All slices used in the experiments were prepared from the livers of phenobarbital-induced rats and cultured for up to 8 h as described in Section 2.3-2.6.

A stock 100 mM BB solution was prepared in DMSO and added to the culture medium such that the final DMSO and BB concentrations were 1% (v/v) and 1 mM respectively. AGE was added directly to the culture medium to achieve final concentrations of 1%, 3% and 5% (v/v). All slices were preincubated in DMEM for 30 min at 37 °C before any experiments were undertaken.

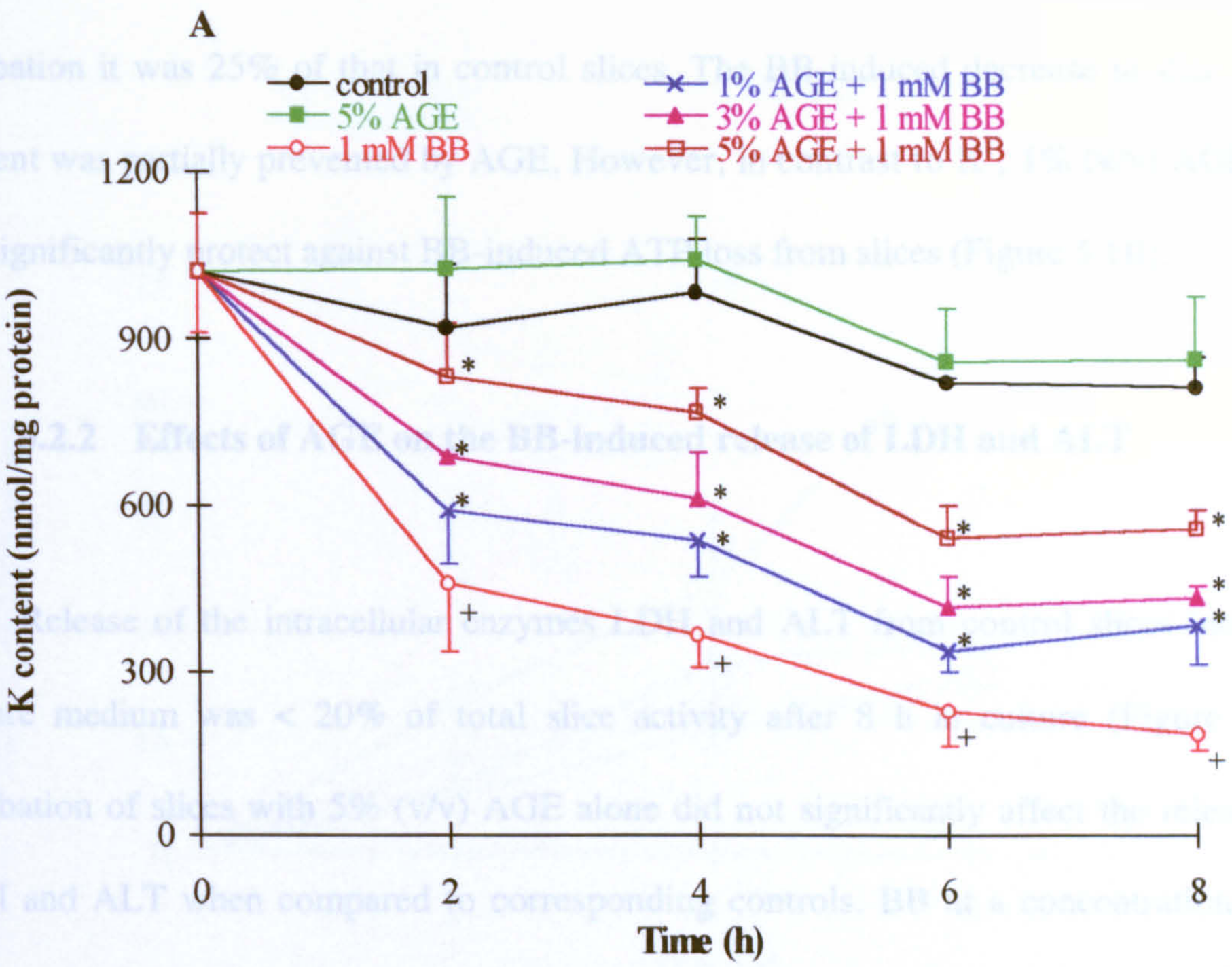
Slices were incubated at 37 °C for up to 8 h and the following parameters of viability determined: loss of intracellular K^+ and ATP, release of LDH and ALT, depletion of NPSH and GSH, and, TBARS formation. All parameters were determined and expressed as described in Section 2.8.

5.2 Results

5.2.1 Effects of AGE on the BB-induced loss of K^+ and ATP

Only modest amounts of K^+ were lost from control slices such that, after 8 h in culture, slice K^+ was still > 80% of that in freshly prepared slices (Figure 5.1A). This modest loss of K^+ from control slices was not affected by the inclusion of 5% (v/v) AGE in the culture medium. BB (1 mM) resulted in a rapid loss of slice K^+ such that, after 8 h in culture, slice K^+ was < 20% of that in freshly prepared slices. When BB-exposed slices were incubated with up to 5% (v/v) AGE for 8 h, a concentration-dependent partial prevention of the loss of slice K^+ was observed. Thus, after 8 h incubation with 1 mM BB, intracellular K^+ was decreased to 17% of that in freshly isolated slices whilst addition of 1%, 3% and 5% (v/v) AGE increased intracellular K^+ to 37%, 43% and 55% of that in freshly isolated slices (Figure 5.1A). These increases were significant ($P < 0.05$) compared to BB alone.

Essentially similar results were obtained for slice ATP content (Figure 5.1B). Thus, after 8 h in culture, slice ATP content was little changed from that of freshly isolated slices. Slice ATP content was dramatically decreased by BB such that after only 2 h



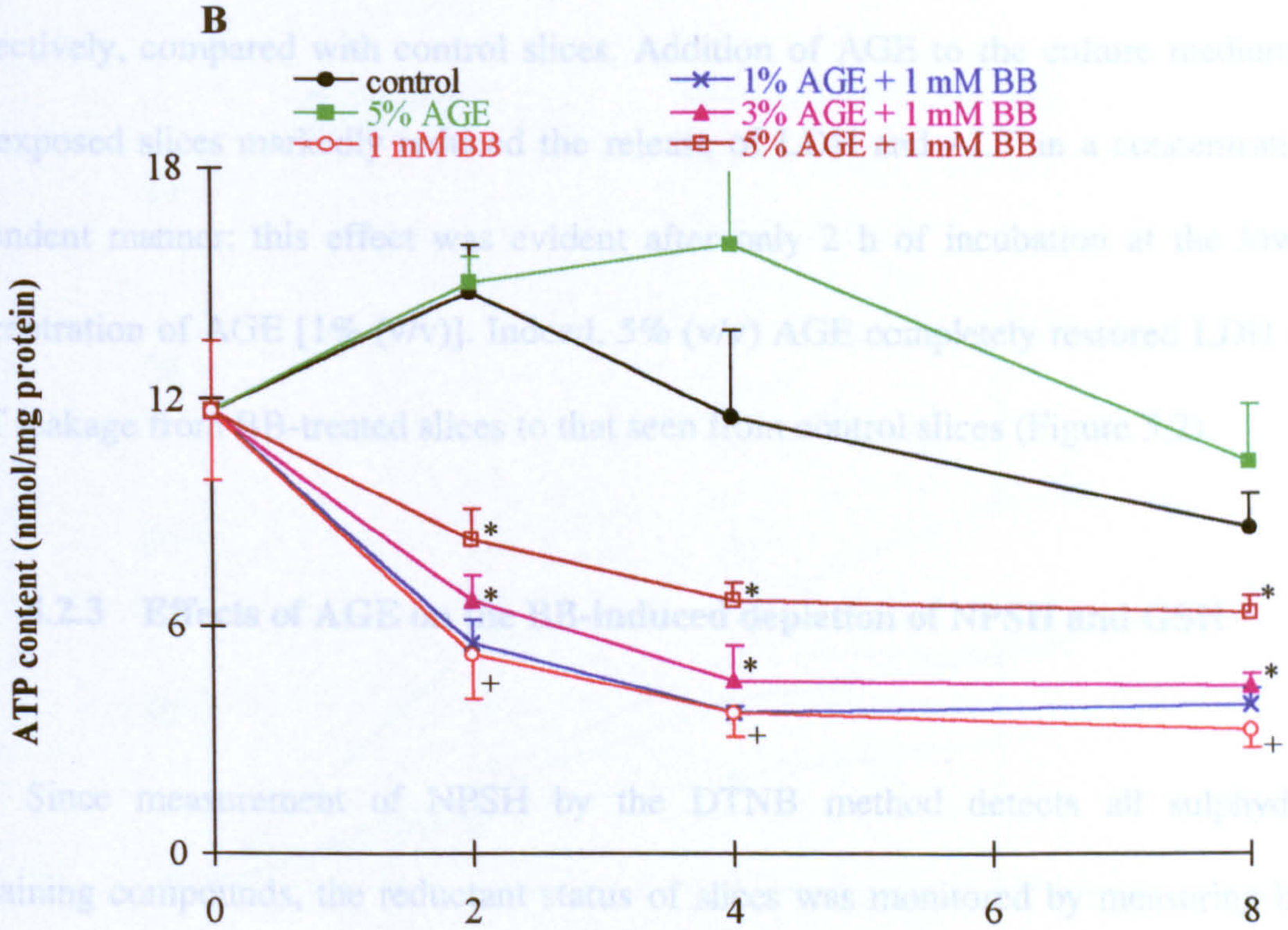


Figure 5.1 Effects of AGE on the BB-induced loss of K^+ (A) and ATP (B) from rat liver slices. Slices were prepared from the livers of phenobarbital-induced animals, preincubated for 30 min and then transferred to fresh medium containing BB and AGE at the concentrations shown. Values are means ± SD from 4 animals. $^{+}P < 0.05$ compared to control, $^{*}P < 0.05$ compared to BB alone.

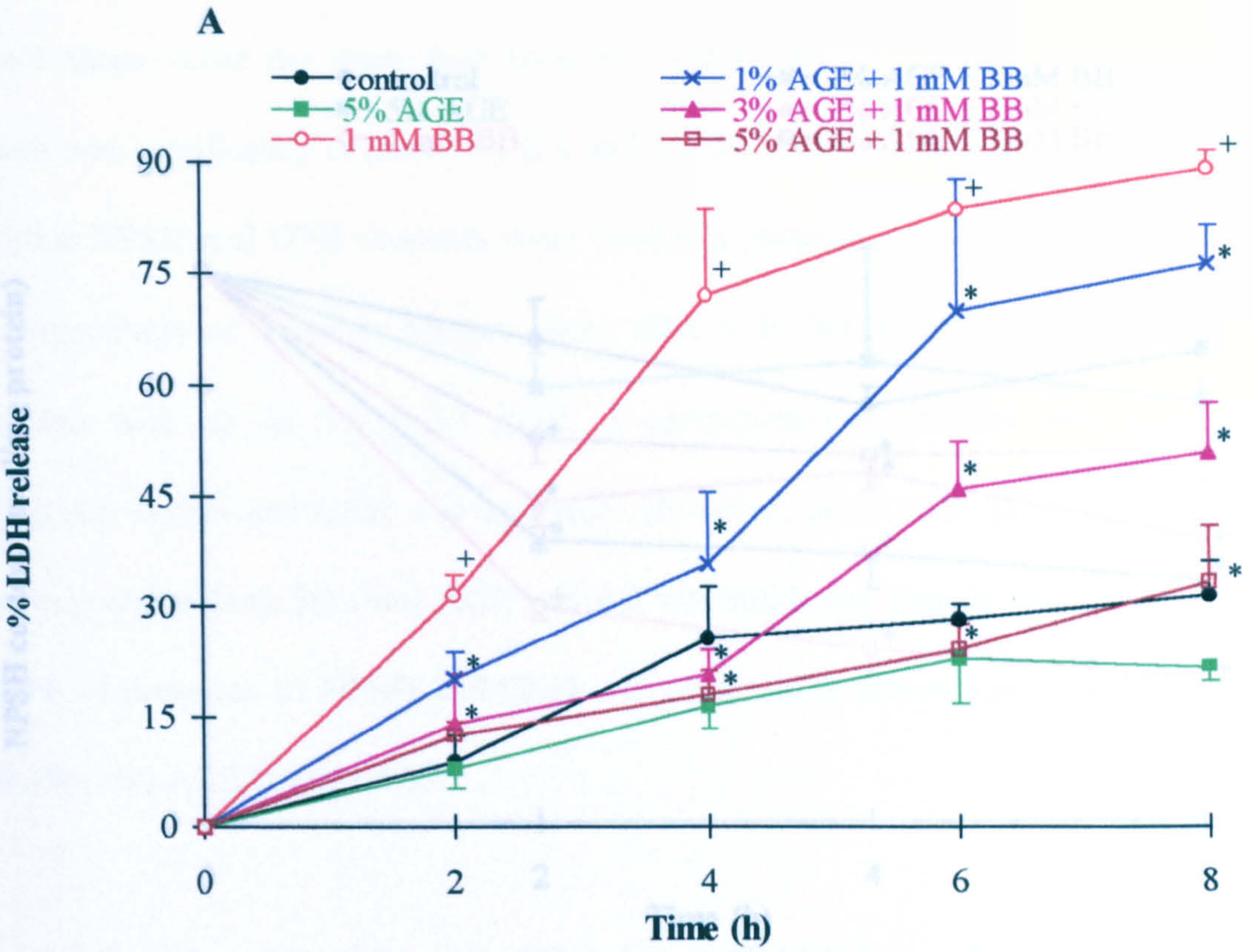
incubation it was 25% of that in control slices. The BB-induced decrease in slice ATP content was partially prevented by AGE. However, in contrast to K^+, 1% (v/v) AGE did not significantly protect against BB-induced ATP loss from slices (Figure 5.1B).

5.2.2 Effects of AGE on the BB-induced release of LDH and ALT

Release of the intracellular enzymes LDH and ALT from control slices into the culture medium was < 20% of total slice activity after 8 h in culture (Figure 5.2). Incubation of slices with 5% (v/v) AGE alone did not significantly affect the release of LDH and ALT when compared to corresponding controls. BB at a concentration of 1 mM significantly increased the release of LDH and ALT by approximately 4- and 2-fold, respectively, compared with control slices. Addition of AGE to the culture medium of BB-exposed slices markedly reduced the release of LDH and ALT in a concentration-dependent manner; this effect was evident after only 2 h of incubation at the lowest concentration of AGE [1% (v/v)]. Indeed, 5% (v/v) AGE completely restored LDH and ALT leakage from BB-treated slices to that seen from control slices (Figure 5.2).

5.2.3 Effects of AGE on the BB-induced depletion of NPSH and GSH

Since measurement of NPSH by the DTNB method detects all sulphydryl-containing compounds, the reductant status of slices was monitored by measuring both NPSH and GSH. The time courses of NPSH and GSH content were similar with all treatments (Figure 5.3). Thus, slice NPSH and GSH declined by 10-20% during 6 h in culture, this was not prevented by inclusion of 5% (v/v) AGE in the culture medium of



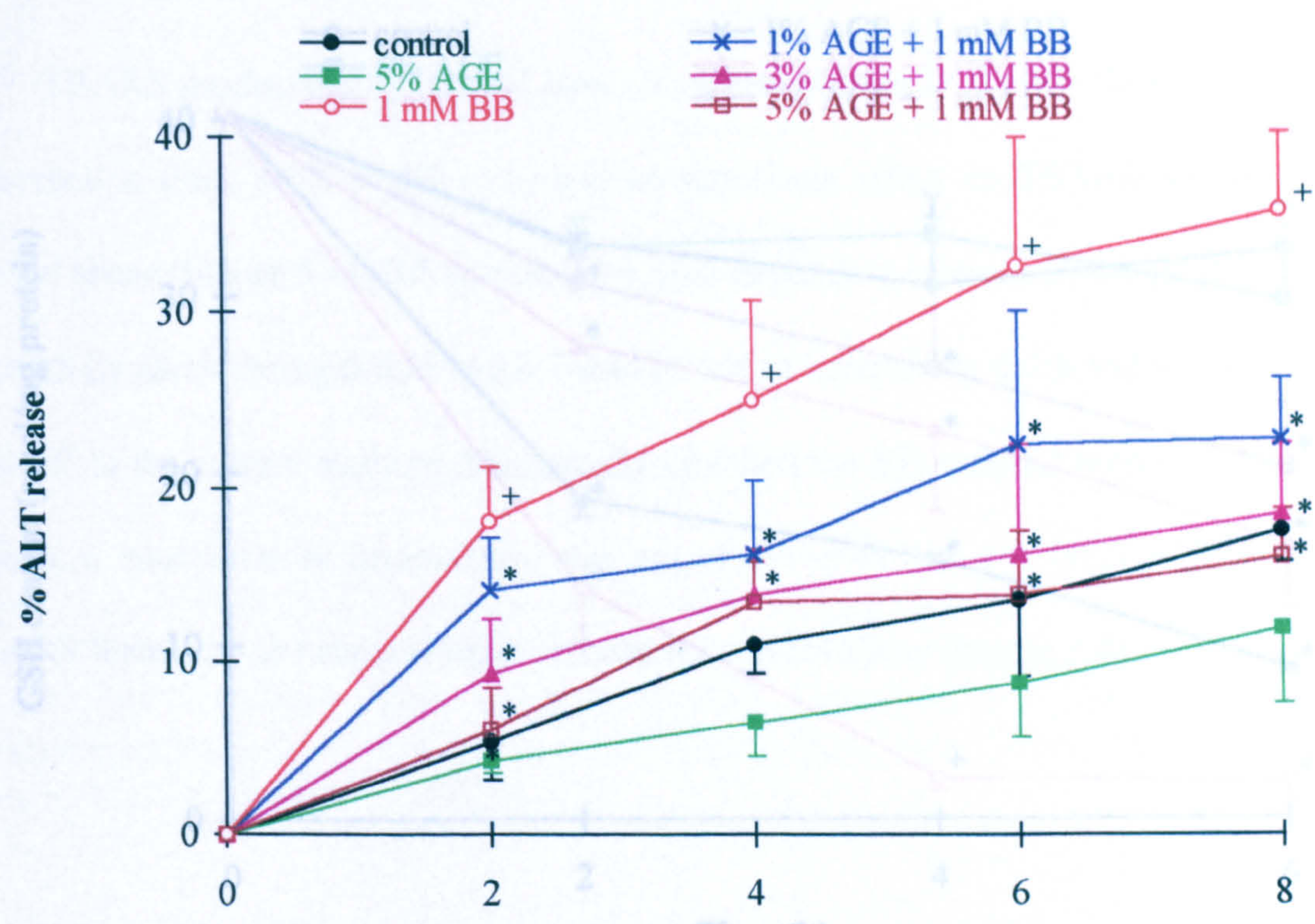
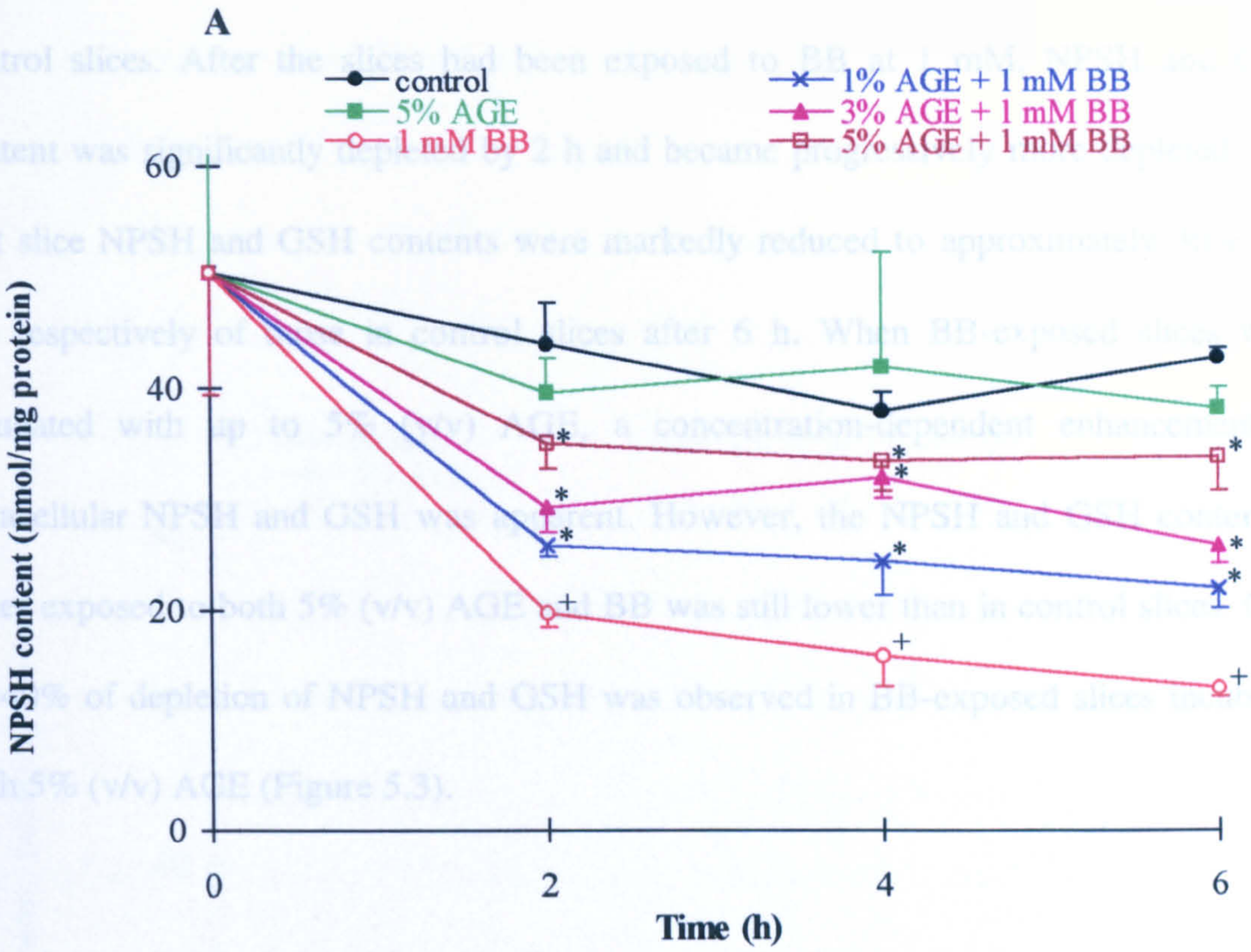


Figure 5.2 Effects of AGE on the BB-induced release of LDH (A) and ALT (B) from rat liver slices. Slices were prepared from the livers of phenobarbital-induced animals, preincubated for 30 min and then transferred to fresh medium containing BB and AGE at the concentrations shown. Values are means ± SD from 6 animals. $^{+}P < 0.05$ compared to control, $^{*}P < 0.05$ compared to BB alone.



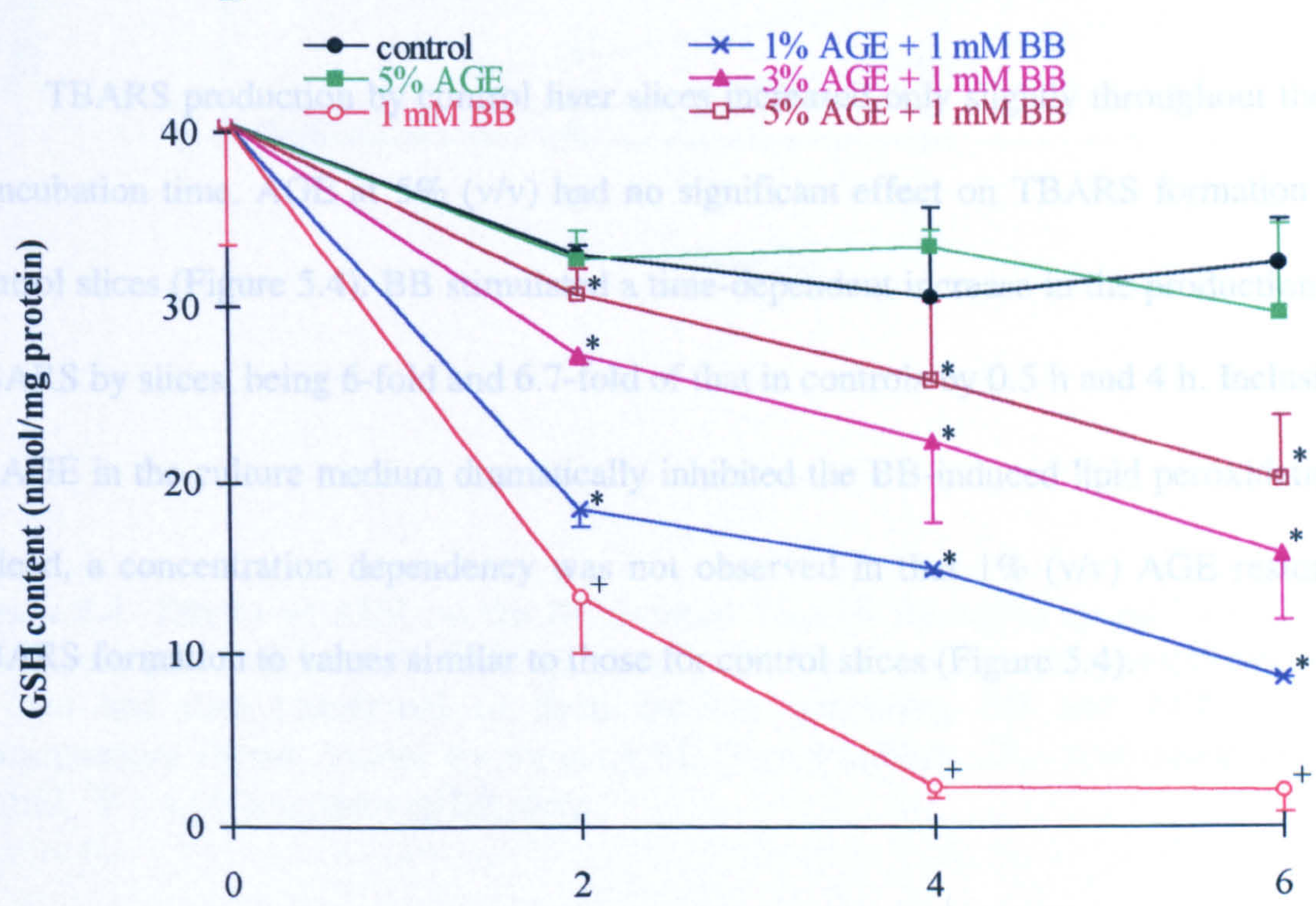


Figure 5.3 Effects of AGE on the BB-induced depletion of NPSH (A) and GSH (B) in rat liver slices. Slices were prepared from the livers of phenobarbital-induced animals, preincubated for 30 min and then transferred to fresh medium containing BB and AGE at the concentrations shown. Values are means ± SD from 6 animals. $^{+}P < 0.05$ compared to control, $^{*}P < 0.05$ compared to BB alone.

control slices. After the slices had been exposed to BB at 1 mM, NPSH and GSH content was significantly depleted by 2 h and became progressively more depleted such that slice NPSH and GSH contents were markedly reduced to approximately 30% and 6% respectively of those in control slices after 6 h. When BB-exposed slices were incubated with up to 5% (v/v) AGE, a concentration-dependent enhancement of intracellular NPSH and GSH was apparent. However, the NPSH and GSH content in slices exposed to both 5% (v/v) AGE and BB was still lower than in control slices. Only 20-40% of depletion of NPSH and GSH was observed in BB-exposed slices incubated with 5% (v/v) AGE (Figure 5.3).

5.2.4 Effects of AGE on the BB-induced TBARS formation

TBARS production by control liver slices increased only slightly throughout the 6 h incubation time. AGE at 5% (v/v) had no significant effect on TBARS formation by control slices (Figure 5.4). BB stimulated a time-dependent increase in the production of TBARS by slices, being 6-fold and 6.7-fold of that in controls by 0.5 h and 4 h. Inclusion of AGE in the culture medium dramatically inhibited the BB-induced lipid peroxidation. Indeed, a concentration dependency was not observed in that 1% (v/v) AGE restored TBARS formation to values similar to those for control slices (Figure 5.4).

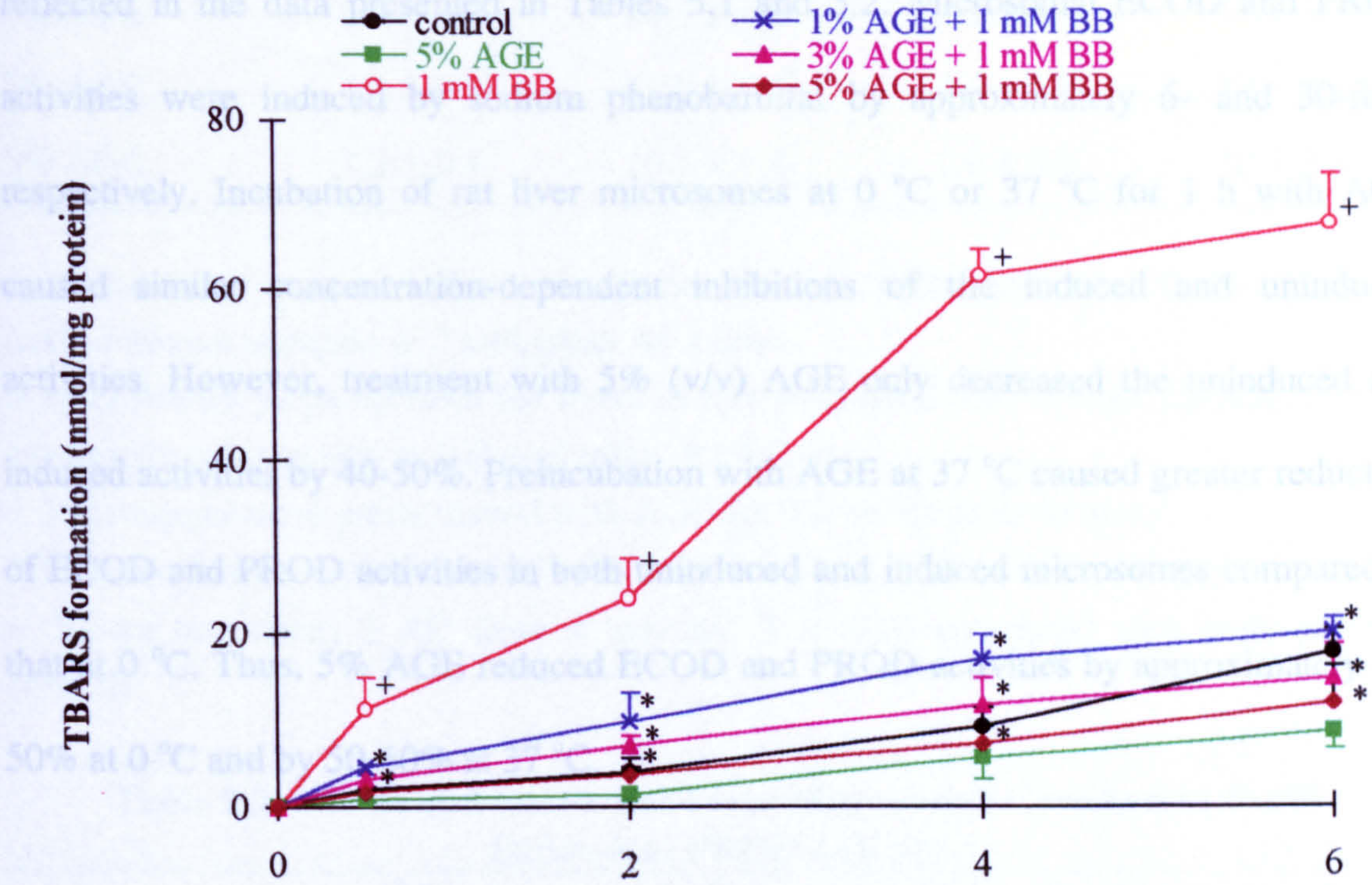


Figure 5.4 Effects of AGE on the BB-induced TBARS formation by rat liver slices. Slices were prepared from the livers of phenobarbital-induced animals, preincubated for 30 min and then transferred to fresh medium containing BB and AGE at the concentrations shown. Values are means ± SD from 4 animals. $^{+}P < 0.05$ compared to control, $^{*}P < 0.05$ compared to BB alone.

5.2.5 Effects of AGE on rat liver microsomal cytochrome P-450 dependent monooxygenase activities

The relative ability of AGE to inhibit ECOD and PROD activities *in* vitro are reflected in the data presented in Tables 5.1 and 5.2. Microsomal ECOD and PROD activities were induced by sodium phenobarbital by approximately 6- and 30-fold, respectively. Incubation of rat liver microsomes at 0 °C or 37 °C for 1 h with AGE caused similar concentration-dependent inhibitions of the induced and uninduced activities. However, treatment with 5% (v/v) AGE only decreased the uninduced and induced activities by 40-50%. Preincubation with AGE at 37 °C caused greater reduction of ECOD and PROD activities in both uninduced and induced microsomes compared to that at 0 °C. Thus, 5% AGE reduced ECOD and PROD activities by approximately 40-50% at 0 °C and by 50-60% at 37 °C.

As mentioned in Section 2.8.12, the experiments to determine ECOD activity in microsomes preincubated with AGE at 0 °C were performed before investigating the linearity of reaction rate with respect to volumes of microsomes added. Volumes of 75 µl was used in these experiments which was above the linear range. As reported in Section 2.8.12, up to 50 µl and 25 µl of microsomes from uninduced and induced rats, respectively, were within the linear range. Thus, the correct volume of microsomes was used in the subsequent experiments. Therefore, as seen in Table 5.1A, ECOD activity in microsomes incubated at 37 °C was considerably higher than that at 0 °C. Even so, the results still showed similar concentration-dependent inhibitions of ECOD activity.

Table 5.1 Effects of AGE on Rat Liver Microsomal Cytochrome P-450 Dependent ECOD Activity

	7-Ethoxycoumarin O-Deethylase (nmol/min/mg protein)[e]			
	0 °C[c]		37 °C[d]	
Group	uninduced[a]	induced[b]	uninduced[a]	induced[b]
Control	2.6 ± 0.4	16.6 ± 1.0	4.2 ± 0.7	19.1 ± 5.0
1% AGE	2.2 ± 0.3*	14.5 ± 1.1*	3.2 ± 0.5*	14.9 ± 3.4*
3% AGE	1.8 ± 0.3*	12.5 ± 1.1*	2.6 ± 0.5*	12.3 ± 2.7*
5% AGE	1.2 ± 0.4*	10.3 ± 1.8*	1.8 ± 0.2*	10.9 ± 2.2*

a. Microsomes prepared from the livers of rats pretreated with 0.9% saline by intraperitoneal injection at 2 ml/kg/day for 4 days.

b. Microsomes prepared from the livers of rats pretreated with sodium phenobarbital by intraperitoneal injection at 100 mg/kg/day for 4 days.

c. Microsomes were preincubated with AGE for 1 h on ice prior to assay.

d. Microsomes were preincubated with AGE for 1 h at 37 °C prior to assay.

e. Values are means ± SD from 4 animals; *P < 0.05 compared with corresponding control.

Table 5.2 Effects of AGE on Rat Liver Microsomal Cytochrome P-450 Dependent PROD Activity

	7-Pentoxyresorufin O-Depentylase (pmol/min/mg protein)[e]			
	0°C[c]		37°C[d]	
Group	uninduced[a]	induced[b]	uninduced[a]	induced[b]
Control	3.8 ± 0.8	109.1 ± 14.5	3.7 ± 0.1	107.9 ± 1.5
1% AGE	3.2 ± 0.7*	86.4 ± 14.0*	2.8 ± 0.1*	75.6 ± 5.0*
3% AGE	2.4 ± 0.5*	67.4 ± 10.2*	2.0 ± 0.1*	56.6 ± 2.2*
5% AGE	1.9 ± 0.4*	59.9 ± 7.7*	1.6 ± 0.1*	47.4 ± 1.6*

a. Microsomes prepared from the livers of rats pre-treated with 0.9% saline by intraperitoneal injection at 2 ml/kg/day for 4 days.

b. Microsomes prepared from the livers of rats pretreated with sodium phenobarbital by intraperitoneal injection at 100 mg/kg/day for 4 days.

c. Microsomes were preincubated with AGE for 1 h on ice prior to assay.

d. Microsomes were preincubated with AGE for 1 h at 37 °C prior to assay.

e. Values are means ± SD from 4 animals; *P < 0.05 compared with corresponding control.

5.3 Discussion

As mentioned in Section 2.1, AGE was formulated in aqueous ethanol. The batches of AGE used in this study contained 8% ethanol. The maximum concentration of AGE used *in vitro* was 5% which is equivalent to 0.4% ethanol (approximately 87 mM) in medium. In retrospect, this concentration of ethanol should have been used in control incubations; this has been omitted in this study.

The results presented in this chapter conclusively demonstrate that AGE has protective effects *in vitro* against BB-induced hepatotoxicity as judged by loss of slice K^+ and ATP, release of LDH and ALT, depletion of NPSH and GSH, and, TBARS formation. The data demonstrated that BB at 1 mM induced significant damage of liver slices as evidenced by decreasing slice K^+, ATP, NPSH and GSH to 10 - 30% of that in corresponding controls, whilst increasing ALT and LDH release by 2- and 4-fold, respectively, and TBARS formation by 6-fold. Loss of the major cellular cation K^+ and the release of intracellular enzymes from the hepatocytes indicate cell membrane damage. The rapid loss of ATP indicates loss of cell function and viability. Addition of 1 -5 % (w/v) AGE to the culture medium significantly protected against cell damage induced by BB and this protection was concentration-dependent.

It is generally accepted that GSH represents the vast majority of total cellular NPSH in liver and, in this study, GSH accounted for approximately 80% of the NPSH in fresh slices. It is noteworthy that 1 mM BB caused almost total depletion of GSH such that, after 4 -6 h in culture, hepatic GSH was decreased by 35 - 40 nmol/mg of protein.

The absolute loss of NPSH was similar (i.e. approximately 35 -40 nmol/mg of protein) such that, after 4 - 6 h in culture, slice NPSH was approximately 15 nmol/mg of protein. Thus, it is clear that BB-induced depletion of NPSH is specific to GSH, and other NPSH compounds are not affected by BB. This strongly suggests that cytochrome P-450-derived metabolites of BB conjugate preferentially to GSH rather than to other NPSH compounds, such as cysteine. AGE exhibited protection against GSH depletion, although even at 5% (v/v) protection by AGE was incomplete. GSH depletion is due largely to its conjugation to the reactive 3,4-oxide formed from BB. It is possible that the mechanism for the protection by AGE against GSH depletion might be that a constituent(s) of AGE reacts preferentially with the 3,4-oxide, thereby sparing slice GSH. Since BB toxicity becomes significant only when GSH is depleted, the liver slices are thus protected. In this study, 5% (v/v) AGE alone did not affect GSH content of slices. Geng and Lau (1997) reported AGE *in vitro* caused both dose- and time-dependent increases in intracellular GSH level in bovine pulmonary artery endothelial cells.

Lipid peroxidation is one of the most important organic expressions of oxidative stress induced by the reactivity of oxygen free radicals (Yagi, 1982). The assessment of MDA has become the most common technique to measure the degree of oxidative damage in biological systems. The most widely employed method for determination of MDA in biological samples is based on its reaction with thiobarbituric acid (Buege and Aust, 1978). Previous studies by Casini *et al.* (1982) demonstrated that in primary cultures of hepatocytes BB induced a rapid depletion of GSH followed by the appearance of lipid peroxidation accompanied by liver cell death. As the major product of lipid peroxidation, MDA was detected by TBARS formation in these studies and BB

stimulated significant production of TBARS. Others workers have also found that BB toxicity in rats *in vivo* and in cultured rat hepatocytes involves the stimulation of lipid peroxidation, detected as an increase in MDA formation (Casini *et al.*, 1982; Locke and Brauer, 1991). Smith *et al.* (1982) reported BB at 0.6 mM produced significant TBARS formation in isolated hepatocytes after 3 -4 h of incubation. Hepatic TBARS formation was increased significantly by the intraperitoneal injection of BB in rats (Park *et al.*, 1996).

In the past few years several investigators have used both *in vivo* and *in vitro* models of liver injury to show that organosulphur compounds of garlic protect hepatocytes from lipid peroxidation induced by various oxidants (Nakagawa *et al.*, 1985, 1989; Kagawa *et al.*, 1986; Horie *et al.*, 1989). As judged by all seven parameters of viability studied, incubation of AGE protected liver slices from BB-induced toxicity. This was dependent on the concentration of AGE for all parameters of viability except TBARS formation. Indeed, TBARS formation was the parameter most sensitive to AGE in that the lowest concentration studied (1%, v/v) completely prevented the BB-induced increase in TBARS formation and hence a concentration dependency was not observed. AGE has also been shown to inhibit TBARS formation in rat liver microsomes exposed to oxidative stress (Imai *et al.*, 1994), in heart homogenates from mice pretreated with doxorubicin (Kojima *et al.*, 1994) and in bovine pulmonary artery endothelial cells exposed to H_2O_2 (Yamasaki *et al.*, 1994). Other workers have also reported the inhibition of TBARS formation in human serum and rat microsomes by garlic powder (Horie *et al.*, 1989; Grune *et al.*, 1996; Park and Choi, 1997). These results also suggest that lipid peroxidation is not the primary cause of BB hepatotoxicity, since 1% (v/v)

AGE offered only marginal protection against BB-induced loss of K^+, ATP, NPSH and GSH, and release of LDH and ALT. Casini *et al.* (1989) concluded that in BB-induced hepatocellular damage, a depletion of GSH may trigger lipid peroxidation, which may also be involved in the pathogenesis of BB intoxication.

Another possible explanation for the protective effect of AGE against BB-induced hepatotoxicity is that the toxic P-450-derived BB metabolites are not formed due to inhibition of some cytochrome P-450 isoforms. Indeed, inhibition of some isoforms of cytochrome P-450 by garlic and garlic organosulphur compounds has been reported (Dalvi, 1992; Gwilt *et al.,* 1994; Reicks and Crankshaw, 1996). Thus, the effect of AGE on ECOD and PROD activities was investigated. ECOD provides an estimate of total P-450 activity whilst PROD is relatively specific for the P-450 isoforms responsible for BB metabolism, namely 2B1 and 2B2 (Gonzalez, 1990). AGE inhibited the induced and non-induced activities of both isoforms to similar extents, implying that it is a relatively non-specific effect. However, even at 5% (v/v) AGE in the assay mixture, the inhibition was approximately 50% at 0 °C and 65% at 37 °C and this cannot explain the almost total reversal of BB toxicity by this concentration of AGE. The partial protection of AGE against BB toxicity is probably due to the small inhibition of P-450 isoforms.

In conclusion, the results presented here have demonstrated clearly that AGE protects against BB-induced toxicity towards rat liver slices *in vitro*. This is most likely to be due to AGE acting as a GSH-sparing agent. It is only possible to speculate upon the active agents involved but they are most likely to be some of the many organosulphur compounds present in AGE and other garlic preparations.

CHAPTER 6

PROTECTIVE EFFECTS OF AGED GARLIC EXTRACT AGAINST BROMOBENZENE TOXICITY *IN VIVO*

After demonstrating in the previous chapter that inclusion of AGE in the incubation medium of precision-cut rat liver slices protects against the toxic effects of bromobenzene, the effects of feeding rats with AGE for 7 days on BB toxicity to subsequently isolated liver slices were investigated . Such a situation mimics more closely the *in vivo* situation.

6.1 Methods

AGE or a 20% (v/v) dilution of AGE were administered orally by stomach tube for 7 days at 10 ml/day/kg body weight. Control animals received dH_2O only. All dosings were performed between 10.00 - 12.00 h and all animals were fasted for 2 h before and 2 h after gavage. Mixed function oxidases were induced by administering sodium phenobarbital to all rats on the last four days of treatment as described in Section 2.2. The slices were prepared and cultured for up to 6 h as described in Sections 2.3 - 2.6.

After 30 min of preincubation in DMEM at 37 °C, slices were transferred to fresh medium to which BB solutions in DMSO had been added to achieve the final desired concentration as described in Section 5.1.

The effects of AGE on BB-induced cytotoxicity were evaluated between 0 - 6 h of incubation by measuring the loss of intracellular K^+ and ATP, release of LDH and ALT, depletion of NPSH and GSH, and, TBARS formation. All parameters were determined and expressed as described in Section 2.8.

6.2 Results

6.2.1 Effects of pretreatment with AGE on the BB-induced loss of K^+ and ATP

Slices from control animals maintained their K^+ content at close to that in freshly-isolated slices for the duration of the 6 h incubation period (Figure 6.1A). Pretreatment of animals with AGE at either dose did not cause significant changes in the K^+ content of slices. BB at 1 mM produced a marked loss of intracellular K^+ from slices from control animals such that after 6 h in culture it was reduced by approximately 75%. This BB-induced loss of K^+ was partially prevented in a dose-dependent manner by pretreatment of animals with AGE (Figure 6.1A).

Essentially similar results were obtained for slice ATP content (Figure 6.1B). The ATP content of slices from control animals was unchanged from that of freshly-isolated slices after 6 h in culture and was not affected by the pretreatment of animals with AGE at either dose used. Slices from control animals incubated with 1 mM BB showed a 60% and 85% reduction in ATP content by 2 h and 6 h, respectively. This dramatic decrease

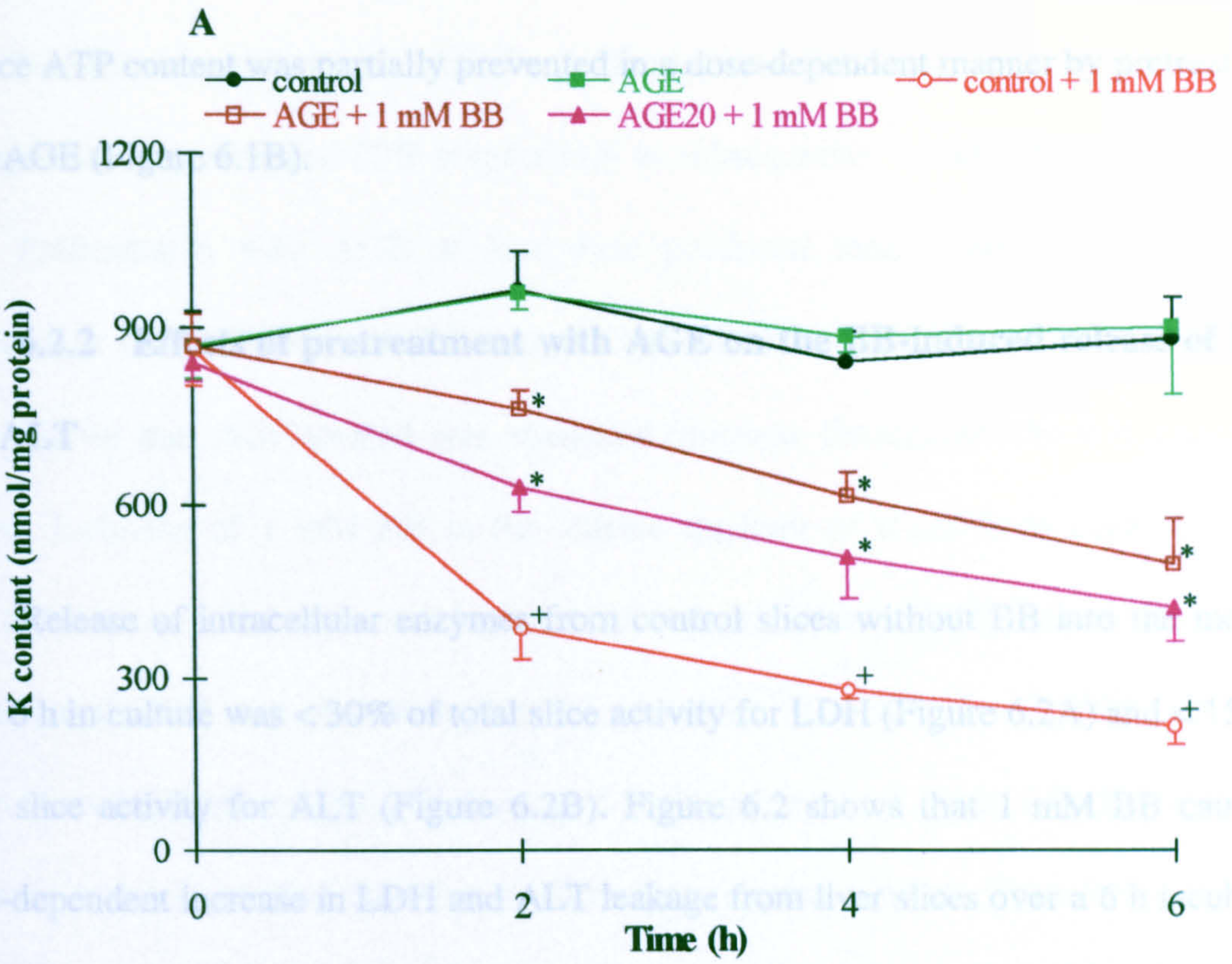


Figure 6.1 Effects of pretreatment with AGE on the BB-induced loss of K^+ (A) and ATP (B) from rat liver slices. Phenobarbital-induced animals were orally dosed with water, AGE or 20% (v/v) of AGE (AGE20) for 7 days at 10ml/day/kg body weight. Subsequently-isolated slices were incubated in the presence or absence of 1 mM BB. Values are means ± SD from 4 animals. $^{+}P < 0.05$ compared to control, $^{*}P < 0.05$ compare to control + 1 mM BB.

in slice ATP content was partially prevented in a dose-dependent manner by pretreatment with AGE (Figure 6.1B).

6.2.2 Effects of pretreatment with AGE on the BB-induced release of LDH and ALT

Release of intracellular enzymes from control slices without BB into the medium after 6 h in culture was < 30% of total slice activity for LDH (Figure 6.2A) and < 15% of total slice activity for ALT (Figure 6.2B). Figure 6.2 shows that 1 mM BB caused a time-dependent increase in LDH and ALT leakage from liver slices over a 6 h incubation period such that an approximately 3-fold increase in LDH and ALT leakage from BB-exposed slices was observed. Pretreatment of animals with AGE to the BB-exposed slices resulted in dose-dependent protective effects against BB-induced enzyme release. Pretreatment with AGE at high dose almost completely restored ALT release in the presence of BB to that seen from control slices (Figure 6.2B). In contrast, partially preventive effects on LDH leakage were observed at both doses used (Figure 6.2A).

6.2.3 Effects of pretreatment with AGE on the BB-induced depletion of NPSH and GSH

The reductant status of slices was monitored by measuring NPSH by the DTNB method together with GSH by a specific enzymatic method.

Pretreatment of rats with AGE for 7 days increased the content of NPSH and GSH by approximately 60% and 80% respectively in subsequently isolated liver slices (Figure 6.3). Pretreatment with AGE at low dose produced more modest, but significant increases in slice NPSH and GSH content. The NPSH and GSH content of slices from both control and AGE-treated rats remained constant throughout the 6 h incubation period. Inclusion of 1 mM BB in the culture medium of slices from control animals resulted in a rapid and extensive depletion of both NPSH and GSH; indeed, almost total depletion of GSH was observed after 6 h in culture. The NPSH and GSH content of slices from AGE-treated rats still decreased in the presence of 1 mM BB. However, this decrease was less marked than that in slices from control animals. Thus, because of the elevated NPSH and GSH contents at time zero, even after 6 h in culture with BB, slices from AGE-treated rats still had NPSH and GSH contents above those of slices from control animals incubated in the absence of BB. Furthermore, after 6 h in culture with BB, slices from rats pretreated with low-dose AGE had NPSH and GSH contents similar to those of slices from control animals.

6.2.4 Effects of pretreatment with AGE on the BB-induced TBARS formation

TBARS formation by slices from control and AGE-treated animals was only 17 nmol/100 mg protein and 11 nmol/100 mg protein after 6 h in culture, respectively (Figure 6.4). BB at 1 mM caused a time-dependent increase in TBARS formation by slices from control animals such that after 6 h in culture TBARS formation increased to approximately 90 nmol/100 mg protein. Pretreatment of animals with AGE completely

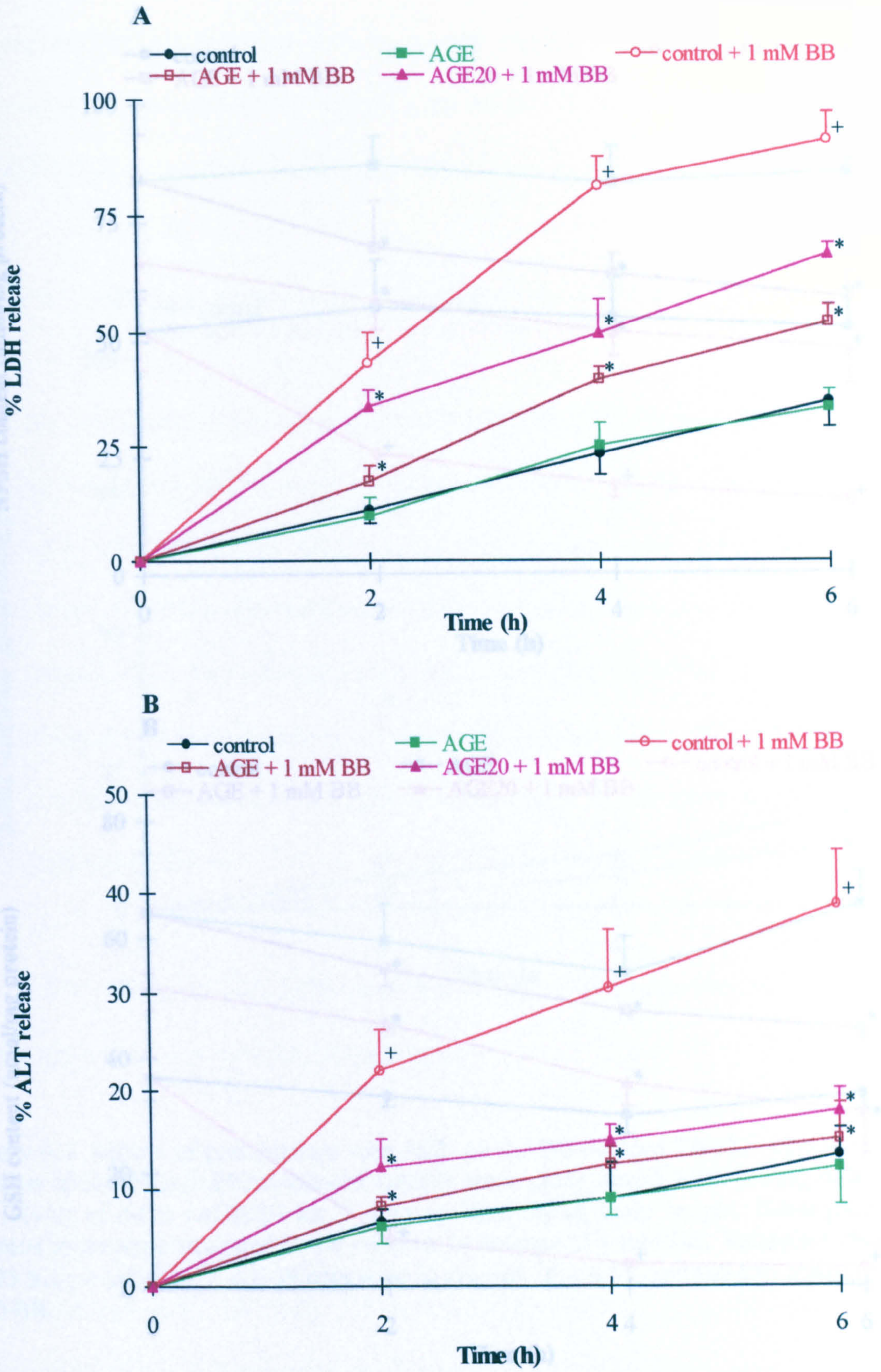


Figure 6.2 Effects of pretreatment with AGE on the BB-induced release of LDH (A) and ALT (B) from rat liver slices. Phenobarbital-induced animals were orally dosed with water, AGE or 20% (v/v) of AGE (AGE20) for 7 days at 10ml/day/kg body weight. Subsequently-isolated slices were incubated in the presence or absence of 1 mM BB. Values are means ± SD from 4 animals. $^{+}P < 0.05$ compared to control, $^{*}P < 0.05$ compared to control + 1 mM BB.

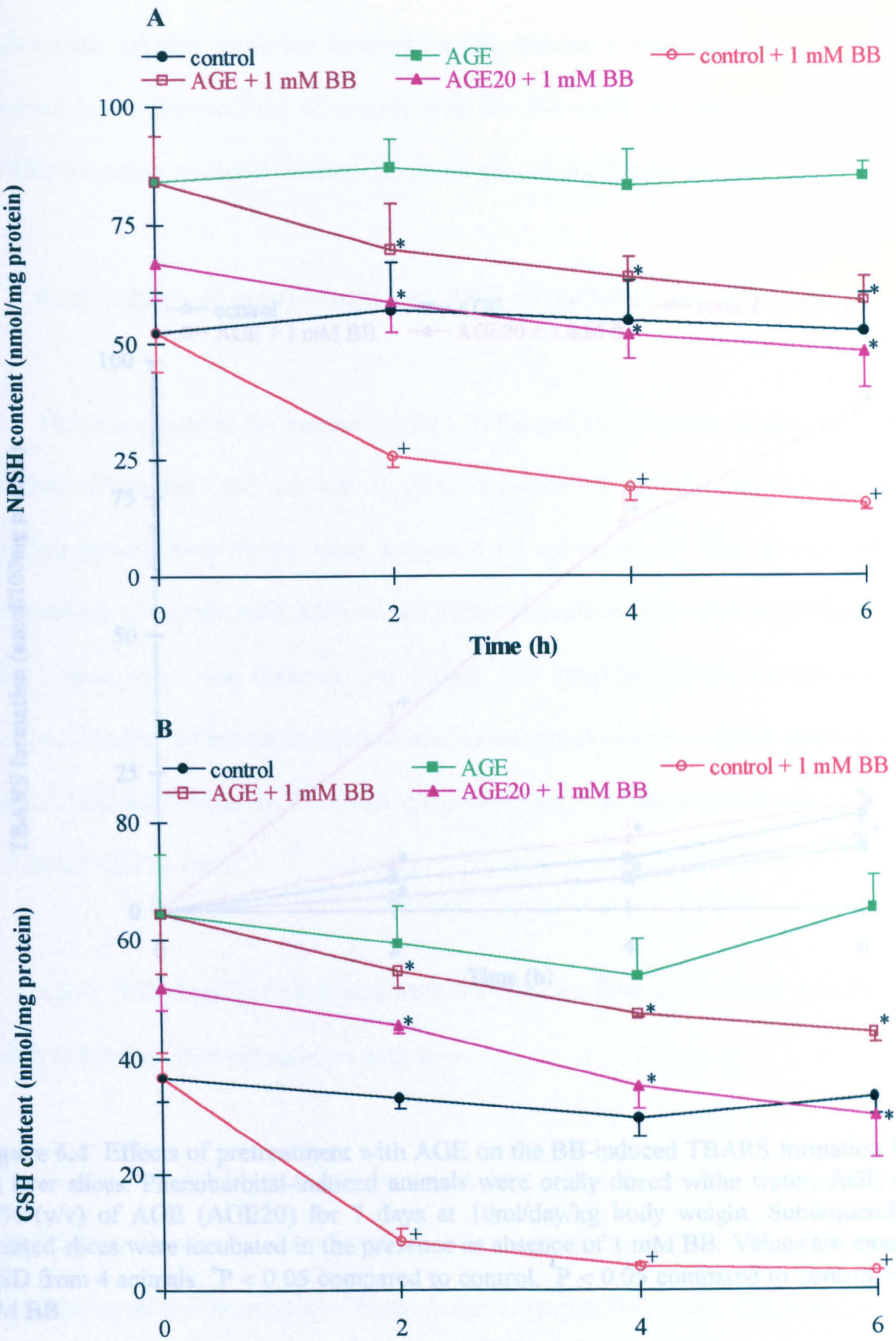


Figure 6.3 Effects of pretreatment with AGE on the BB-induced depletion of NPSH (A) and GSH (B) in rat liver slices. Phenobarbital-induced animals were orally dosed with water, AGE or 20% (v/v) of AGE (AGE20) for 7 days at 10ml/day/kg body weight. subsequently-isolated slices were incubated in the presence or absence of 1 mM BB. Values are means ± SD from 4 animals. $^{+}P < 0.05$ compared to control, $^{*}P < 0.05$ compared to control + 1 mM BB.

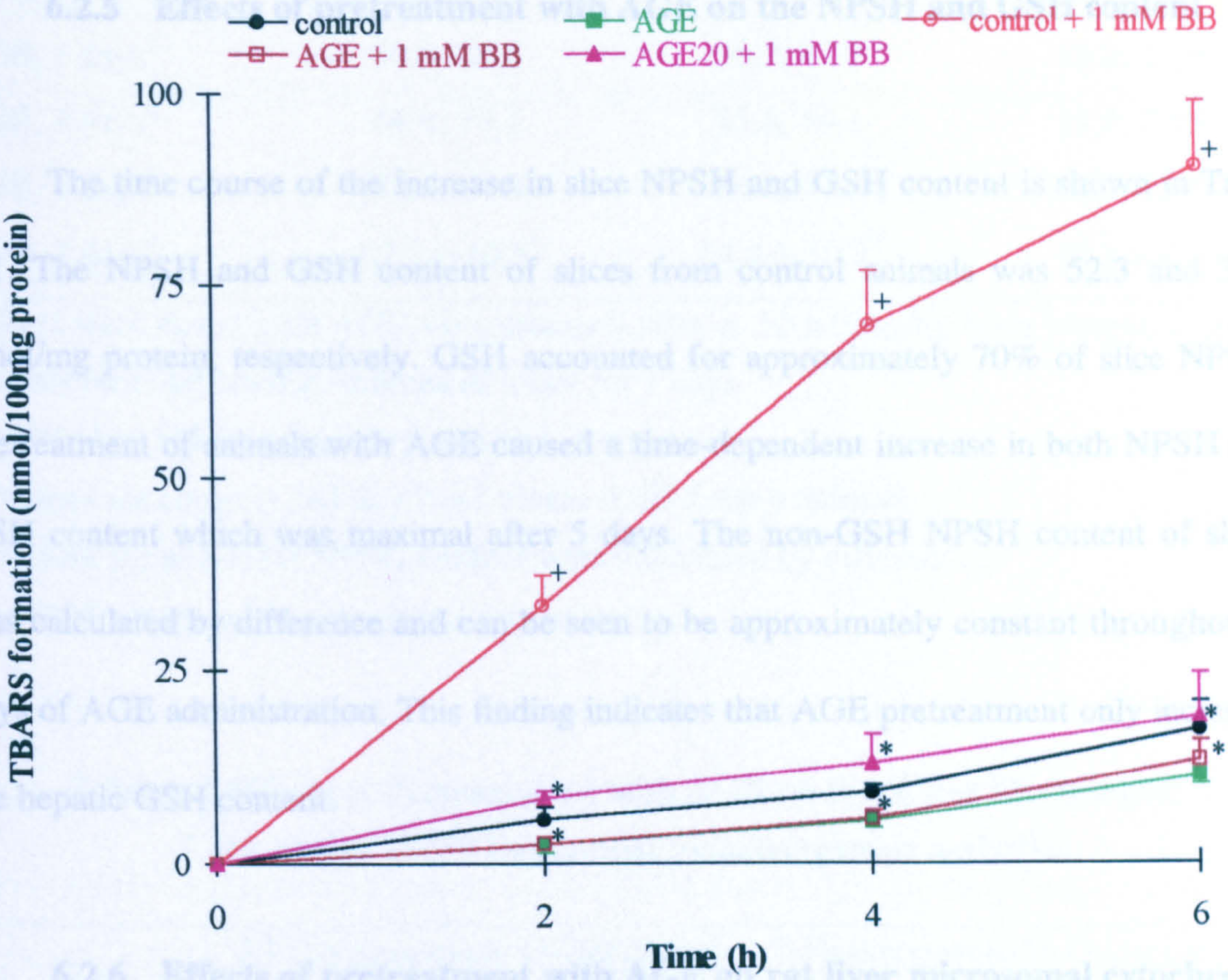


Figure 6.4 Effects of pretreatment with AGE on the BB-induced TBARS formation by rat liver slices. Phenobarbital-induced animals were orally dosed withe water, AGE or 20% (v/v) of AGE (AGE20) for 7 days at 10ml/day/kg body weight. Subsequently-isolated slices were incubated in the presence or absence of 1 mM BB. Values are means ± SD from 4 animals. $^{+}P < 0.05$ compared to control, $^{*}P < 0.05$ compared to control + 1 mM BB.

inhibited the TBARS formation induced by BB. Indeed, a dose dependency was not observed in that pretreatment of animals with the 20% (v/v) dilution of AGE restored TBARS formation to values similar to those by slices from control animals (Figure 6.4).

6.2.5 Effects of pretreatment with AGE on the NPSH and GSH content

The time course of the increase in slice NPSH and GSH content is shown in Table 6.1. The NPSH and GSH content of slices from control animals was 52.3 and 36.7 nmol/mg protein, respectively. GSH accounted for approximately 70% of slice NPSH. Pretreatment of animals with AGE caused a time-dependent increase in both NPSH and GSH content which was maximal after 5 days. The non-GSH NPSH content of slices was calculated by difference and can be seen to be approximately constant throughout 7 days of AGE administration. This finding indicates that AGE pretreatment only increases the hepatic GSH content.

6.2.6 Effects of pretreatment with AGE on rat liver microsomal cytochrome P-450 dependent monooxygenase activities

ECOD and PROD activities in microsomes from the livers of phenobarbital-induced control animals were 10.4 ± 0.6 nmol/min/mg protein and 62.8 ± 4.7 pmol/min/mg protein respectively. Pretreatment of animals for 7 days with both doses of AGE did not affect either enzyme activity in subsequently isolated microsomes (Table 6.2).

Table 6.1 Effects of Pretreatment with AGE on the NPSH and GSH Content of Rat Liver Slices

Treatment[a]	NPSH content (nmol/mg protein)	GSH content (nmol/mg protein)	Non-GSH NPSH content[d] (nmol/mg protein)
Control[c]	52.3 ± 8.6	36.7 ± 4.2	16.4 ± 8.5
AGE, 1 days[b]	50.0, 65.6	36.2, 47.0	13.8, 18.6
AGE, 3 days[b]	64.4, 79.1	45.6, 59.1	18.8, 20.0
AGE, 5 days[b]	82.6, 84.9	64.4, 61.3	18.2, 23.6
AGE, 7 days[c]	85.2 ± 10.5*	64.4 ± 10.2*	17.8 ± 4.5

a. Rats were dosed with AGE via a stomach tube at 10 ml/day/kg body weight. Control animals received similar volumes of water for 7 days.

b. Individual values are given for days 1, 3 and 5.

c. Values for controls and day 7 are means ± SD from 4 animals.

d. Values for non-GSH NPSH content were calculated by difference.

*P < 0.05 compared to corresponding controls.

Table 6.2 Effects of Pretreatment with AGE on Rat Liver Microsomal Cytochrome P-450 Dependent monooxygenase activities

Group[a]	7-Ethoxycoumarin O-Deethylase (nmol/min/mg protein)[b]	7-Pentoxyresorufin O-Depentylase (pmol/min/mg protein)[b]
Control	10.4 ± 0.6	62.8 ± 4.7
AGE	10.8 ± 0.5	65.6 ± 6.4
20% AGE	10.6 ± 1.1	63.9 ± 2.5

a. Rats were dosed with AGE or a 20% (v/v) dilution of AGE via a stomach tube at 10 ml/day/kg body weight for 7 days. Control animals received similar volumes of water. Mixed function oxidases were induced by administering sodium phenobarbital by intraperitoneal injection at 100 mg/kg/day employing 0.9% saline (2 ml/kg) as the injection vehicle to all rats on the last four days of treatment.

b. Values are means ± SD from 5 animals.

6.3 Discussion

In order to mimic more closely the *in vivo* situation where garlic would be ingested orally on a regular basis, the effects of feeding rats with AGE for 7 days on BB toxicity to subsequently isolated precision-cut liver slices have been investigated. Slice K^+, ATP, NPSH and GSH, LDH and ALT release, and, the TBARS formation have been used as indicators of toxicity. In addition, the effects of feeding AGE on the hepatic content of GSH and on some cytochrome P-450 activities were also monitored.

Pretreatment of rats with AGE for 7 days resulted in a dose-dependent protective effect against BB-induced toxicity to liver slices. All the indicators of toxicity studied showed that liver slices from AGE-treated rats are resistant to BB toxicity and this is more pronounced at the higher of the two doses administered. Thus, the BB-induced leakage of K^+, loss of ATP and leakage of the two intracellular enzymes LDH and ALT were all reduced by pretreatment with AGE. However, lipid peroxidation, as judged by the formation of TBARS, was reduced to control levels even at the lower dose of AGE. This suggests that lipid peroxidation is a secondary event of BB toxicity.

The detoxifying effects of garlic and related organosulphur compounds could be related to their ability to inhibit phase I enzymes, induce phase II enzymes, or bind to exogenous toxins through sulphydryl groups (Abdullah *et al.*, 1988; Dausch and Nixon, 1990). Singh and Rao (1995) evaluated and confirmed a significant modulation in the activities of phase I and phase II enzymes, -SH content, and MDA levels in mice by garlic. Several papers have reported prevention of liver damage caused by carbon

tetrachloride; for example, in cultured hepatocytes by garlic oil, S-allyl cysteine and S-methyl cysteine (Hikino *et al.*, 1986), by AGE administered to mice (Nakagawa *et al.*, 1989) and by garlic oil administered to rats and mice (Kim *et al.*, 1995). AGE has been reported to protect liver microsomal membranes from lipid peroxidation (Horie *et al.*, 1989; Park and Choi, 1997). Wang *et al.* (1996) also demonstrated that fresh garlic homogenate and its organosulphur compounds have time- and dose-dependent protective effect against paracetamol-induced hepatotoxicity in mice.

A possible explanation for the protective effect of AGE is that the toxic P-450-derived metabolites of BB are not formed due to inhibition of some cytochrome P-450 isoforms. This cannot be the case here since both ECOD and PROD activities were similar in microsomes isolated from livers of control and AGE-treated rats. ECOD activity estimates total cytochrome P-450 whilst PROD is relatively specific for those isoforms responsible for BB metabolism, namely 2B1 and 2B2 (Gonzalez, 1990). Although garlic and some organosulphur compounds inhibit some isoforms of P-450, such effects are limited to the oil-soluble organosulphur compounds (Reicks and Crankshaw, 1996). Indeed, diallyl sulphide has been shown to inhibit cytochrome P-450 2E1 (Gwilt *et al.*, 1994; Hu *et al.*, 1996a).

Since GSH has been shown to play a critical role in the hepatotoxicity of BB, of more interest is the substantial increase in the hepatic content of NPSH and GSH by pretreatment of AGE. This effect occurred over several days and was specific to glutathione in that the hepatic content of other non-GSH NPSH (presumably mainly free cysteine) was not increased by AGE pretreatment. Similar increases in GSH content have

been shown in both bovine pulmonary artery endothelial cells after 24 h in culture with AGE (Geng and Lau, 1997) and in human prostate carcinoma cells after 3 h in culture with S-allyl cysteine and S-allyl mercaptocysteine (Pinto *et al.*, 1997). However, it must be emphasised that in the previous chapter, the GSH content of rat liver slices was not increased after 6 h in culture with up to 5% (v/v) AGE. Since the primary mode of detoxification of the 3,4-oxide metabolite of BB is by conjugation with GSH, an increased hepatic content of GSH would be expected to confer a higher capacity for detoxification.

The overall effect of AGE on GSH metabolism in liver slices is still unknown. The mechanism by which pretreatment of rats with AGE increases the hepatic content of GSH must involve either enhanced synthesis and/or decreased utilisation. It is doubtful that decreased glutathione S-transferase activity could account for decreased utilisation; indeed, the garlic constituents diallyl sulphide, diallyl trisulphide (Sparnins *et al.*, 1988; Hu *et al.*, 1996b) and S-allyl cysteine (Hatono *et al.*, 1996) have been shown to induce some isoforms of glutathione S-transferase in liver. Specific garlic extracts have been shown to affect sulphydryl-disulphide exchange reactions that may involve both oxidised glutathione and reduced glutathione (Sparnins *et al.*, 1988; Gudi and Singh, 1991; Scharfenberg *et al.*, 1994). Alternatively, an increase in the bioavailability of cysteine might increase GSH synthesis since this is usually the limiting substrate. Such a mechanism has been proposed for N-acetyl cysteine, a hepatoprotective agent used widely to treat paracetamol poisoning (Rafeiro *et al.*, 1994). Thus, hepatic concentrations of cysteine increase dramatically 10 min after intraperitoneal injection of N-acetyl cysteine into rats (Yao *et al.*, 1994). In addition, it has been reported that the

hepatoprotective effect of N-acetyl cysteine is blocked by the GSH synthesis inhibitor buthionine sulphoximine (Miners *et al.*, 1984; Rafeiro *et al.*, 1994), and it is not reproduced by the non-physiological stereoisomer N-acetyl D-cysteine (Corcoran and Wong, 1986). This suggests that N-acetyl cysteine is deacetylated relatively easily. If the organosulphur compounds in AGE are acting in a similar manner here, they must also be metabolised to yield free cysteine. However, the observations that the NPSH content of rat liver slices does not increase following incubation with AGE (see Chapter 5), nor do hepatic concentrations of non-GSH NPSH increase after treatment of rats with AGE, do not support this hypothesis.

It is interesting to note that in slices from AGE-treated rats, not only was the initial hepatic content of GSH elevated, but the GSH content fell less on exposure to BB. Thus, slices from control rats lost approximately 35 nmol GSH/mg of protein over 6 h whilst slices from rats treated with both high-dose and low-dose AGE lost only approximately 20 nmol GSH/mg of protein. It is well established that in cases where xenobiotic metabolism generates electrophilic metabolites leading to depletion of GSH, administration of some thiol-containing compounds may decrease toxicity, either by facilitating GSH synthesis or by sparing GSH as a result of direct formation of conjugates with the electrophilic species (Thor *et al.*, 1979). Although garlic and commercial garlic preparations contain many organosulphur compounds which are nucleophilic, none of those identified carry free sulphydryl groups. The possibility that these are generated by metabolism cannot be eliminated, but is unlikely here since treatment of rats with AGE did not increase the hepatic content of non-GSH NPSH. It is most likely that any GSH sparing effect exerted by the organosulphur compounds in

AGE is due to their nucleophilic nature allowing them to directly trap electrophilic species such as BB-3,4-oxide. Such a situation must occur *in vitro* since the hepatic content of GSH was not increased when liver slices were incubated for 6 h in the presence of 5% (v/v) AGE, although protection against BB-induced toxicity and GSH depletion was still observed (see Chapter 5).

As judged by the releases of ALT and LDH and slice K^+ and ATP contents, the protection against BB toxicity by AGE was incomplete even at the highest dose used. However, slices from rats pretreated with AGE exposed to BB had GSH content greater than untreated controls. This implies that some BB toxicity cannot be abolished by conjugation with GSH and that some toxic species of BB do not interact with GSH.

In summary, the results presented in this chapter demonstrate that AGE has time- and dose-dependent protective effects against BB-induced hepatotoxicity *in vivo*. It would appear that the mechanism of the protective effect of AGE against BB hepatotoxicity involves two possibly interrelated components. The first, which is seen both in liver slices from AGE-treated rats and when AGE is added to liver slices *in vitro*, represents a GSH sparing effect, presumably due to conjugation of organosulphur compounds in AGE with the 3,4-oxide of BB. The second, which is seen only after pretreating animals with AGE, is a slow process occurring over several days and leads to a specific elevation in the GSH content of liver. The GSH-sparing effect would be expected to decrease the utilisation of GSH and hence may explain the long-term elevation of hepatic GSH content.

CHAPTER 7

PROTECTIVE EFFECTS OF S-ALLYL CYSTEINE AGAINST BROMOBENZENE TOXICITY *IN VITRO*

The results of Chapters 5 and 6 show that AGE exerts a protective effect against BB toxicity both *in vitro* and *in vivo*. The active compounds of garlic preparations are believed to be their organosulphur constituents and one of the most abundant of these in AGE is SAC. Thus, the ability of SAC included in the incubation medium of rat liver slices to prevent toxicity caused by BB was investigated.

7.1 Methods

Animals were administered with sodium phenobarbital as described in Section 2.2. All slices used in these experiments were prepared from the livers of phenobarbital-induced rats and cultured for up to 6 h as described in Sections 2.3 - 2.6.

BB solutions were prepared in DMSO and added to the culture medium as described in Section 5.1. SAC was dissolved in dH_2O and added directly to the culture medium to achieve final concentrations of up to 10 mM. Slices were preincubated in DMEM for 30 min at 37 °C before any experiments were undertaken.

The effects of SAC on BB-induced cytotoxicity were evaluated between 0 - 6 h of culture by measuring the loss of K^+, release of LDH and ALT, depletion of NPSH, and,

TBARS formation. All parameters were determined and expressed as described in Section 2.8.

7.2 Results

7.2.1 Effects of SAC on the BB-induced loss of K^+

The K^+ content of control slices was maintained at close to that in freshly-isolated slices for up to 6 h in culture (Figure 7.1). SAC at concentrations of 0.01 - 10 mM did not affect slice K^+ content. For the sake of clarity, only data for 10 mM SAC are shown in Figure 7.1; similar results were obtained for 0.01, 0.1 and 1 mM SAC. A time-dependent decrease in slice K^+ was observed with 1 mM BB; this was highly significant as early as 2 h, and by 6 h 80% of slice K^+ was lost. Inclusion of SAC at concentrations of 0.1 - 10 mM partially prevented the BB-induced loss of K^+ from cultured slices in a concentration-dependent manner whilst SAC at 0.01 mM had little or no protective effect (Figure 7.1).

7.2.2 Effects of SAC on the BB-induced release of LDH and ALT

The release of LDH and ALT from control slices into the medium after 6 h in culture was approximately 25% and 15% of total slice activity, respectively (Figure 7.2 & 7.3). SAC at 0.01 - 10 mM did not significantly affect the release of enzymes from the control slices during the 6 h incubation (only data for 10 mM SAC is shown in Figure 7.2 & 7.3). BB at 1 mM led to approximately 90% of LDH and 40% of ALT activity being released into the medium. When the BB-exposed slices were also treated with SAC at

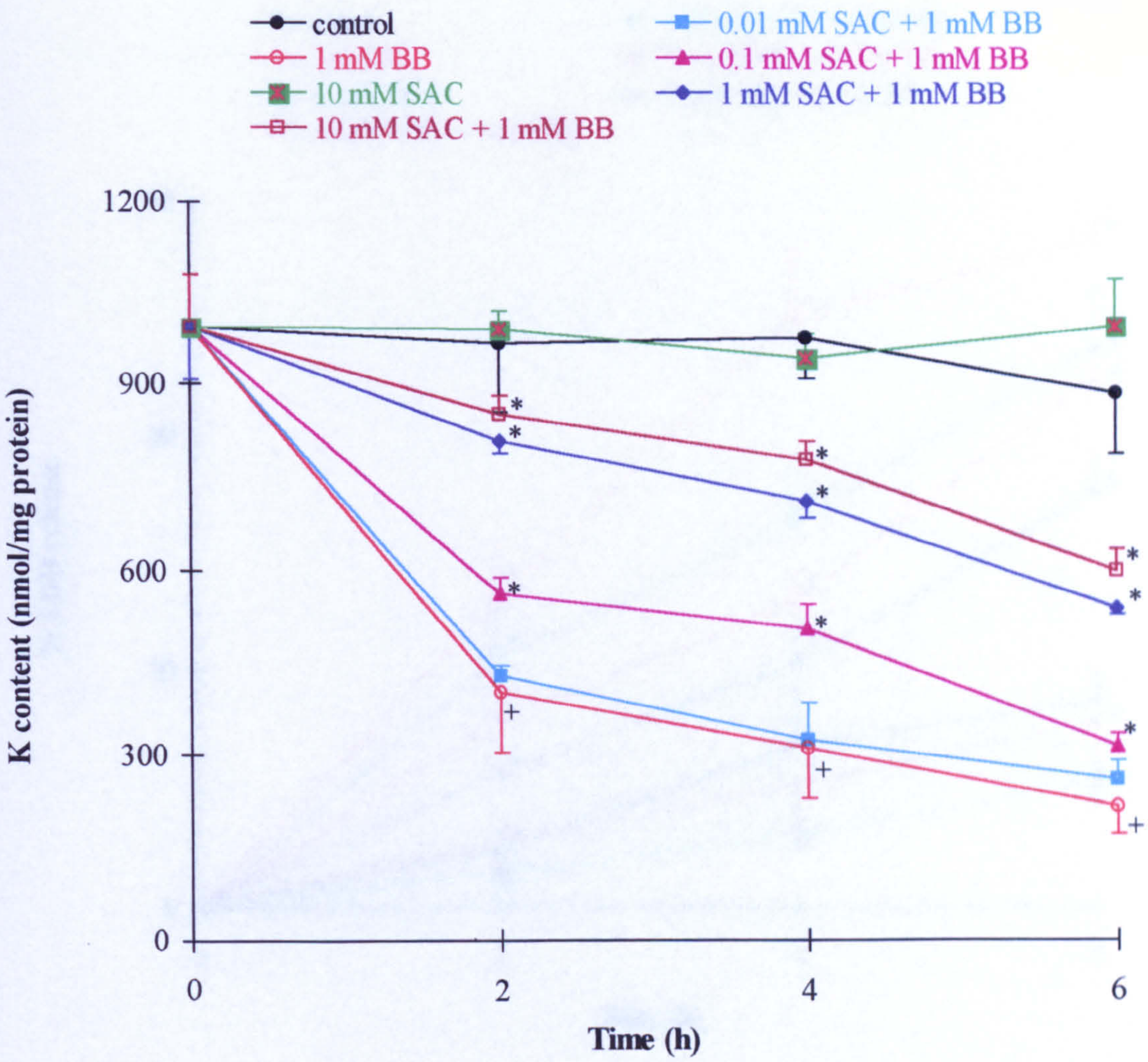


Figure 7.1 Effects of SAC on the BB-induced loss of K^+ from rat liver slices. Slices were prepared from the livers of phenobarbital-induced animals, preincubated for 30 min and then transferred to fresh medium containing BB and SAC at the concentrations shown. Values are means ± SD from 4 animals. $^{+}P < 0.05$ compared to control, $^{*}P < 0.05$ compared to BB alone.

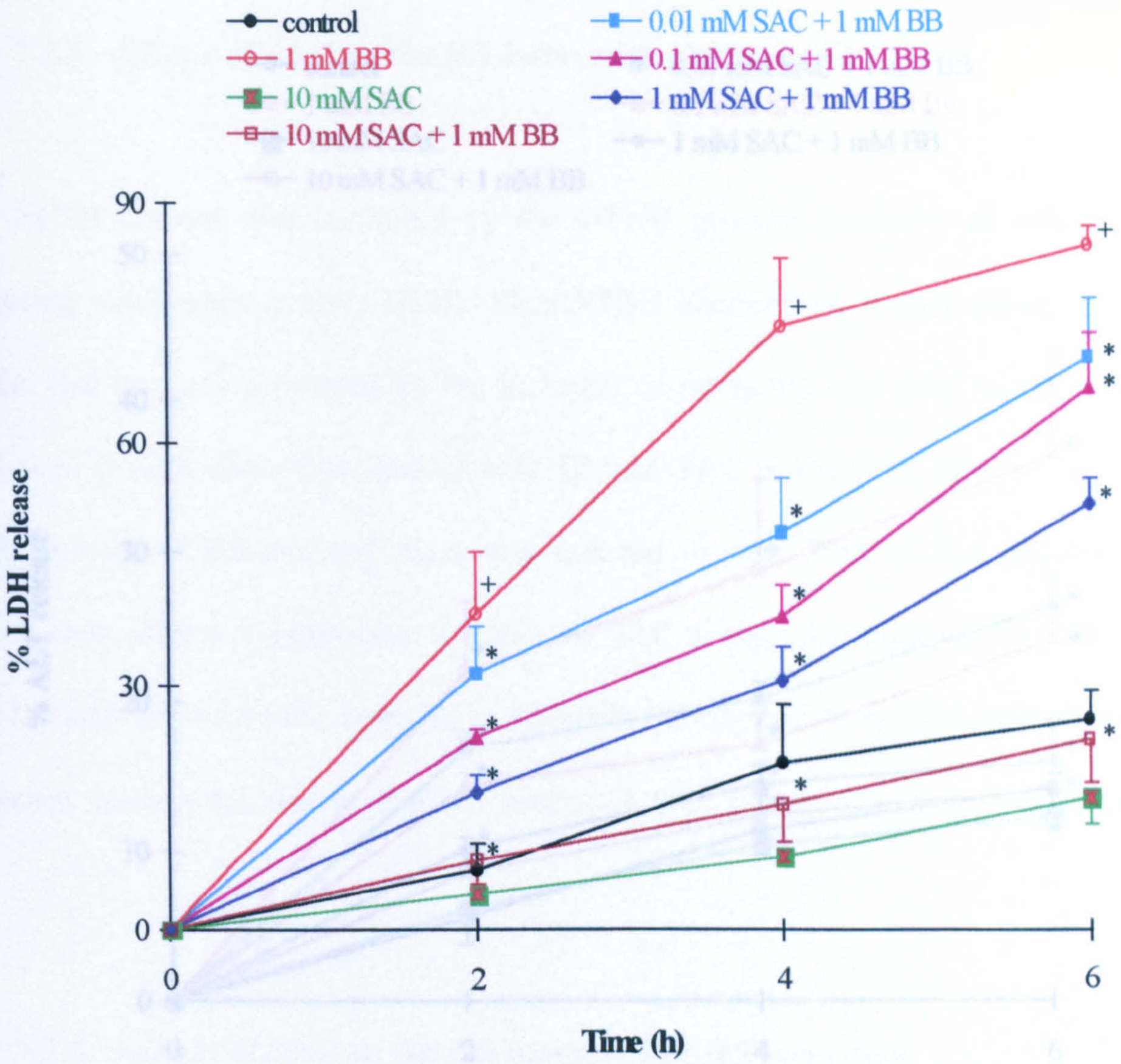


Figure 7.2 Effects of SAC on the BB-induced release of LDH from rat liver slices. Slices were prepared from the livers of phenobarbital-induced animals, preincubated for 30 min and then transferred to fresh medium containing BB and SAC at the concentrations shown. Values are means ± SD from 4 animals. $^{+}P < 0.05$ compared to control, $^{*}P < 0.05$ compared to BB alone.

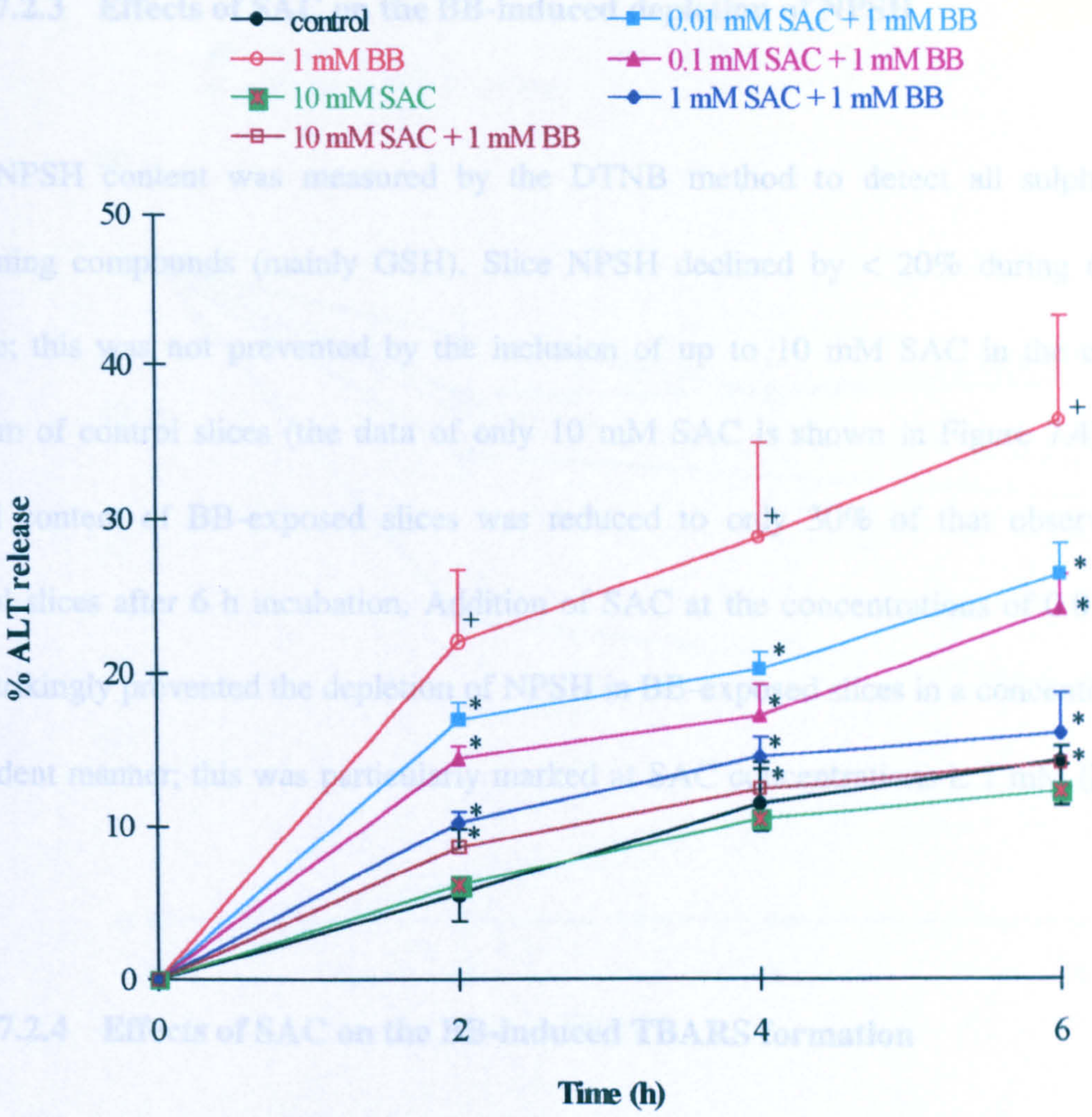


Figure 7.3 Effects of SAC on the BB-induced release of ALT from rat liver slices. Slices were prepared from the livers of phenobarbital-induced animals, preincubated for 30 min and then transferred to fresh medium containing BB and SAC at the concentrations shown. Values are means ± SD from 4 animals. $^{+}P < 0.05$ compared to control, $^{*}P < 0.05$ compared to BB alone.

concentrations of 0.01 - 10 mM, the leakage of both enzymes was markedly reduced in a concentration-dependent manner. Indeed, 10 mM SAC reduced leakage of LDH and ALT from BB-treated slices to the levels seen in control slices (Figure 7.2 & 7.3).

7.2.3 Effects of SAC on the BB-induced depletion of NPSH

NPSH content was measured by the DTNB method to detect all sulphydryl-containing compounds (mainly GSH). Slice NPSH declined by < 20% during 6 h in culture; this was not prevented by the inclusion of up to 10 mM SAC in the culture medium of control slices (the data of only 10 mM SAC is shown in Figure 7.4). The NPSH content of BB-exposed slices was reduced to only 30% of that observed in control slices after 6 h incubation. Addition of SAC at the concentrations of 0.01 - 10 mM strikingly prevented the depletion of NPSH in BB-exposed slices in a concentration-dependent manner; this was particularly marked at SAC concentrations ≥ 1 mM (Figure 7.4).

7.2.4 Effects of SAC on the BB-induced TBARS formation

The release of TBARS into the incubation medium increased slightly from control slices during 6 h incubation (Figure 7.5). This increase in TBARS formation from control slices was not affected by the inclusion of up to 10 mM SAC in the culture medium. BB significantly increased TBARS formation by slices by approximately 5-fold. A dramatic suppression of TBARS formation from BB-exposed slices was observed by inclusion of

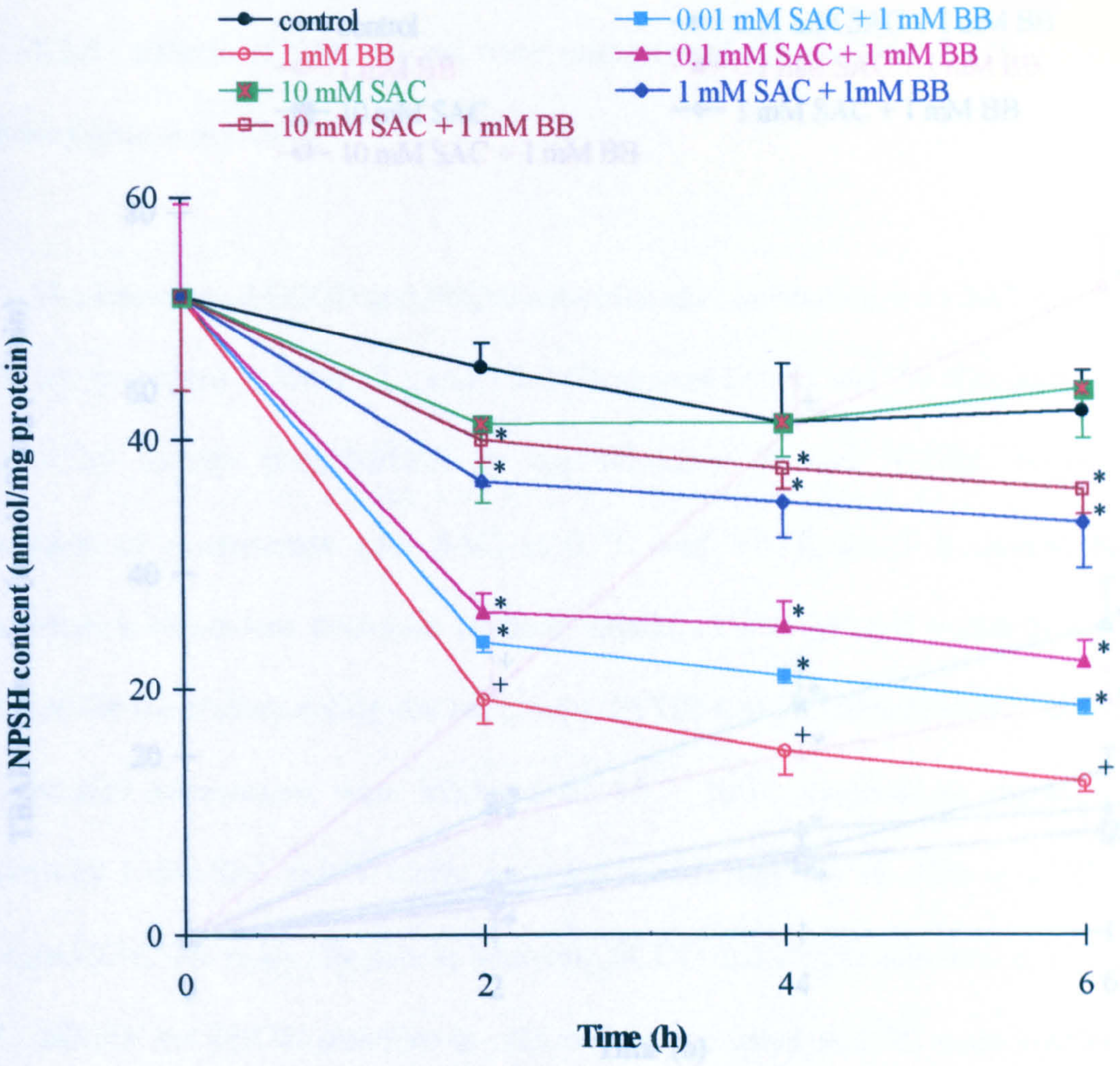


Figure 7.4 Effects of SAC on the BB-induced depletion of NPSH in rat liver slices. Slices were prepared from the livers of phenobarbital-induced animals, preincubated for 30 min and then transferred to fresh medium containing BB and SAC at the concentrations shown. Values are means ± SD from 4 animals. $^{+}P < 0.05$ compared to control, $^{*}P < 0.05$ compared to BB alone.

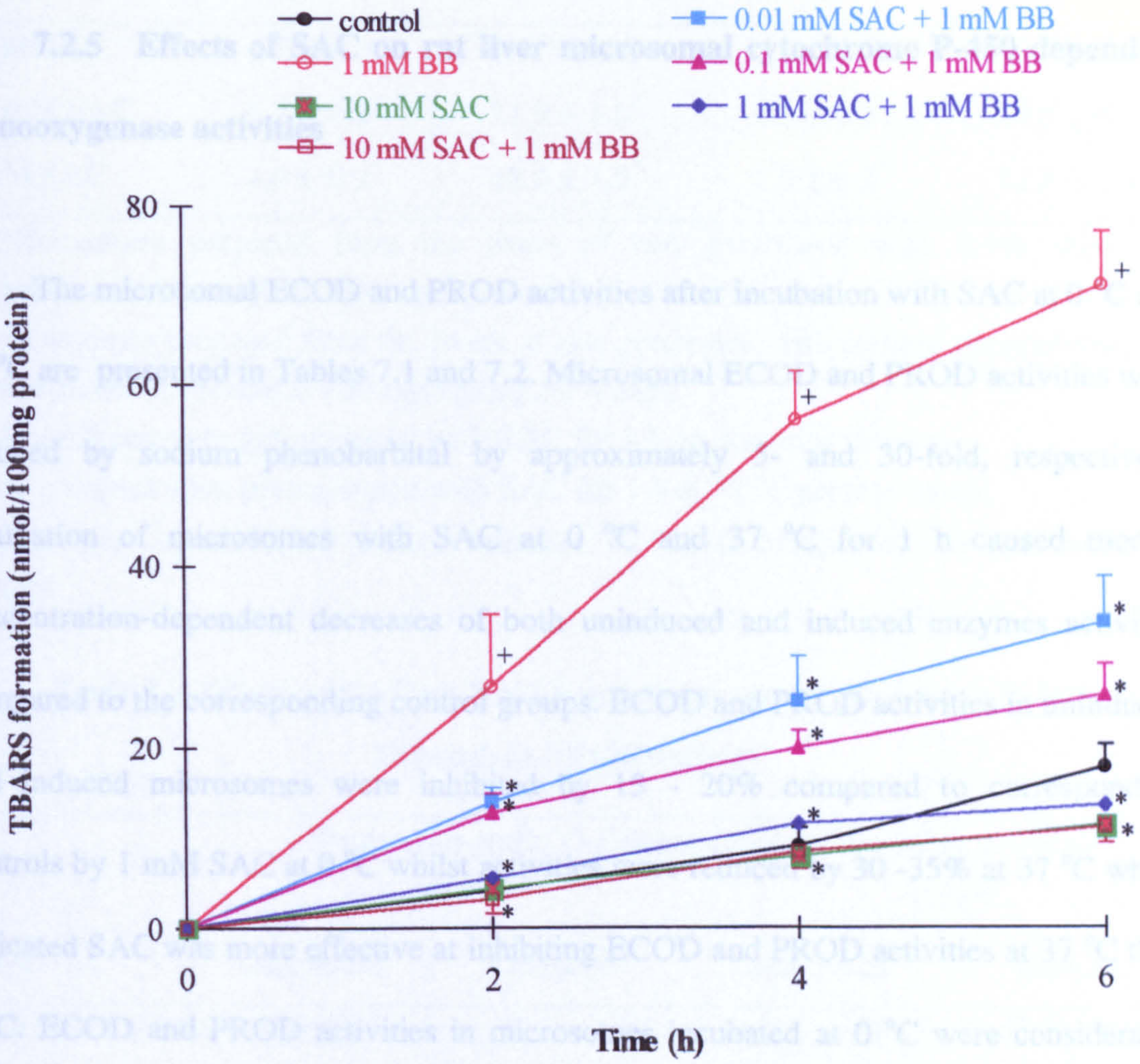


Figure 7.5 Effects of SAC on the BB-induced TBARS formation by rat liver slices. Slices were prepared from the livers of phenobarbital-induced animals, preincubated for 30 min and then transferred to fresh medium containing BB and SAC at the concentrations shown. Values are means ± SD from 4 animals. $^{+}P < 0.05$ compared to control, $^{*}P < 0.05$ compared to BB alone.

SAC in the culture medium. A concentration dependency was observed in addition of SAC at lower concentrations such that ≥ 1 mM SAC restored TBARS formation to values similar to those of control slices (Figure 7.5).

7.2.5 Effects of SAC on rat liver microsomal cytochrome P-450 dependent monooxygenase activities

The microsomal ECOD and PROD activities after incubation with SAC at 0 °C and 37 °C are presented in Tables 7.1 and 7.2. Microsomal ECOD and PROD activities were induced by sodium phenobarbital by approximately 5- and 30-fold, respectively. Incubation of microsomes with SAC at 0 °C and 37 °C for 1 h caused modest concentration-dependent decreases of both uninduced and induced enzymes activities compared to the corresponding control groups. ECOD and PROD activities in uninduced and induced microsomes were inhibited by 15 - 20% compared to corresponding controls by 1 mM SAC at 0 °C whilst activities were reduced by 30 -35% at 37 °C which indicated SAC was more effective at inhibiting ECOD and PROD activities at 37 °C than 0 °C. ECOD and PROD activities in microsomes incubated at 0 °C were considerably higher than that determined in microsomes incubated at 37 °C for both uninduced and induced groups (Table 7.1 & 7.2). That indicates that ECOD and PROD activities declined faster during incubation at 37 °C in comparison with 0 °C.

Table 7.1 Effects of SAC on Rat Liver Microsomal Cytochrome P-450 Dependent ECOD activity

	7-Ethoxycoumarin O-Deethylase (nmol/min/mg protein)[e]			
	0 °C[c]		37 °C[d]	
Group	uninduced[a]	induced[b]	uninduced[a]	induced[b]
Control	5.0 ± 0.3	24.0 ± 1.2	4.4 ± 0.2	18.8 ± 0.6
0.1 mM SAC	4.7 ± 0.2*	21.9 ± 1.0*	3.6 ± 0.1*	14.9 ± 0.7*
1 mM SAC	4.0 ± 0.3*	18.6 ± 1.9*	2.9 ± 0.2*	12.8 ± 0.4*

a. Microsomes prepared from the livers of rats pretreated with 0.9% saline by intraperitoneal injection at 2 ml/kg/day for 4 days.

b. Microsomes prepared from the livers of rats pretreated with sodium phenobarbital by intraperitoneal injection at 100 mg/kg/day for 4 days.

c. Microsomes were preincubated with SAC for 1 h on ice prior to assay.

d. Microsomes were preincubated with SAC for 1 h at 37 °C prior to assay.

e. Values are means ± SD from 4 animals; *P < 0.05 compared with corresponding control.

Table 7.2 Effects of SAC on Rat Liver Microsomal Cytochrome P-450 Dependent PROD activity

	7-Pentoxyresorufin O-Depentylase (pmol/min/mg protein)[e]			
	0° C[c]		37 °C[d]	
Group	uninduced[a]	induced[b]	uninduced[a]	induced[b]
Control	3.9 ± 0.6	122 ± 2.8	2.7 ± 0.3	88.0 ± 9.2
0.1 mM SAC	3.7 ± 0.3*	112 ± 3.9*	2.2 ± 0.2*	72.0 ± 5.9*
1 mM SAC	3.1 ± 0.2*	104 ± 2.6*	1.9 ± 0.2*	58.7 ± 3.2*

a. Microsomes prepared from the livers of rats pretreated with 0.9% saline by intraperitoneal injection at 2 ml/kg/day for 4 days.

b. Microsomes prepared from the livers of rats pretreated with sodium phenobarbital by intraperitoneal injection at 100 mg/kg/day for 4 days.

c. Microsomes were preincubated with SAC for 1 h on ice prior to assay.

d. Microsomes were preincubated with SAC for 1 h at 37 °C prior to assay.

e. Values are means ± SD from 4 animals; *P < 0.05 compared with corresponding control.

7.3 Discussion

The results presented in Chapters 5 and 6 demonstrated that AGE protects against BB toxicity both when it is added to slices *in vitro* or fed to rats for 7 days prior to the preparation of slices. Since SAC is the most abundant sulphur compound in AGE and since the SAC content is used to standardise commercial AGE, the potential hepatoprotective effects towards rat liver slices *in vitro* have been studied by monitoring slice K^+, release of LDH and ALT, depletion of NPSH, and TBARS formation. In addition, the *in vitro* effect of SAC on some microsomal P-450 activities was also investigated.

Concentration- and time-dependent protective effects of SAC against BB-induced hepatotoxicity were observed. All parameters of toxicity were substantially affected by 1 mM BB whereas SAC on its own had no effect. As judged by all five parameters of toxicity studied, addition of SAC to the culture medium protected rat liver slices from BB-induced toxicity. This protection was dependent on the concentration of SAC for all parameters of viability. TBARS formation was reduced to control levels at 1 mM of SAC and hence a concentration dependency was only observed at the lower concentrations added to culture medium. NPSH depletion caused by BB was also significantly reduced by the inclusion of SAC in culture medium, although SAC even at 10 mM did not restore the NPSH concentrations completely. The NPSH content of SAC-treated slices was similar to those in control slices. These data differ from the results of Li *et al.* (1995) and Pinto *et al.* (1997) who found that the GSH content of human breast cancer cells and prostate carcinoma cells was elevated by SAC.

As described in Section 1.5, GSH depletion is due to its conjugation to the highly toxic BB-3,4-oxide formed by cytochrome P-450 from BB. The conjugation with GSH is catalysed by glutathione S-transferase. The other metabolite is the less toxic 2,3-oxide which is subsequently detoxified through hydrolysis by epoxide hydrase. Thus, a likely explanation for the protection against GSH depletion is that SAC reacts preferentially with the 3,4-oxide, thereby sparing slice GSH. Since BB toxicity becomes significant only when GSH is depleted the liver slices are thus protected. However, the possibility cannot be excluded that SAC may also modulate other detoxification enzyme systems, such as glutathione S-transferase or epoxide hydrase which have been demonstrated by Li *et al.* (1995), Hatono *et al.* (1996) and Pinto *et al.* (1997).

The effects of SAC on ECOD and PROD activities were determined. ECOD provides an estimate of total P-450 activity whilst PROD is relatively specific for the P-450 isoforms responsible for BB metabolism, namely P-450 2B1 and 2B2 (Gonzalez, 1990). SAC inhibited the induced and non-induced activities of both isoforms to similar extents, implying that it is a relatively non-specific effect. The inhibition of AGE or SAC on ECOD and PROD activities seemed to be more effective when microsomes were incubated with AGE or SAC at 37 °C in comparison with 0 °C. Glockner and Muller (1995) reported ECOD activity in hand-made slices declined faster during incubation at 37 °C in comparison with 30 °C. A possible explanation for the protective of SAC against BB-induced hepatotoxicity is likely that the toxic P-450-derived BB metabolites are not formed due to inhibition of some isoforms of cytochrome P-450. However, even at 1 mM SAC in the assay mixture, the inhibition was only 20-35% and this cannot explain the almost total reversal of some indicators of BB toxicity by this concentration

of SAC, but it may partially explain the effect of SAC against BB-induced hepatotoxicity by inhibition of some cytochrome P-450 isoforms.

The concentration of SAC in 1% (v/v) AGE is approximately 0.1 mM. An approximately 50% inhibition of ECOD and PROD activities were observed by 5% AGE (Table 5.1). This indicates that there are some organosulphur compounds other than SAC in AGE also involved in the inhibitory effect of P-450 activity. The results of all five parameters of viability studied show that the degree of protection by 0.1 mM SAC against BB-induced toxicity to rat liver slices was similar to that observed in slices incubated with 1% AGE. In conclusion, the results presented here demonstrate that SAC is a key and abundant active constituent in AGE which protects against BB-induced hepatotoxicity towards rat liver slices *in vitro*.

CHAPTER 8

PROTECTIVE EFFECTS OF N-ACETYL CYSTEINE AGAINST BROMOBENZENE TOXICITY *IN VITRO*

N-acetyl cysteine (NAC) is a modulator of thiol levels that protects against hepatotoxic agents, particularly paracetamol. The mechanism of toxicity of paracetamol shows some similarities to that of BB. The hepatotoxicity of paracetamol is caused by a chemically reactive metabolite, N-acetyl-p-benzoquinone imine (NAPQI), which is generated primarily in the liver by a number of cytochrome P-450 enzymes including CYP1A2, CYP2E1 and CYP3A4 (Park, 1995). NAPQI is normally detoxified by conjugation with GSH whilst at higher doses of paracetamol, GSH depletion occurs and NAPQI accumulates. Subsequently, NAPQI binds to macromolecules in the hepatocytes causing cell necrosis (Prescott and Critchely, 1983). NAC is widely used clinically as the treatment for paracetamol overdose. In this chapter the protective properties of NAC against BB toxicity using cultured rat liver slices were investigated.

8.1 Methods

Animals were administered sodium phenobarbital as described in Section 2.2. All slices used in these experiments were prepared from the livers of phenobarbital-induced rats and cultured for up to 6 h as described in Sections 2.3 - 2.6.

BB was dissolved in DMSO and added to the culture medium such that the final concentrations of BB and DMSO were 1 mM and 1% (v/v) respectively. NAC was dissolved in dH_2O and added directly to the culture medium to achieve the final desired concentrations. All slices were preincubated in DMEM for 30 min at 37 °C before any experiments were undertaken.

The effects of NAC on BB-induced hepatotoxicity were detected for up to 6 h in culture by measuring the loss of K^+, release of LDH and ALT, depletion of NPSH, and, TBARS formation. All parameters were determined and expressed as described in Section 2.8.

8.2 Results

8.2.1 Effects of NAC on the BB-induced loss of K^+ and depletion of NPSH

Control slices maintained their K^+ and NPSH content similar to that measured in freshly-isolated slices for the duration of the 6 h incubation period (Figure 8.1A & B). NAC at 10 mM in culture medium did not alter the K^+ content of control slices. In contrast, a modest increase in NPSH content by 10 mM NAC was observed after 4 h incubation and was maintained after 6 h. BB at 1 mM caused a rapid loss of intracellular K^+ and NPSH such that, after 6 h in culture, K^+ and NPSH were < 30% of that in freshly prepared slices. Addition of NAC at concentrations of 0.1 - 10 mM exhibited a pronounced concentration-dependent inhibition of the BB-induced loss of K^+ and depletion of NPSH. The loss of K^+ from BB-exposed slices was partially prevented by

inclusion of 10 mM NAC while slice NPSH was completely restored to those values in control slices by the same concentration of NAC (Figure 8.1A & B).

8.2.2 Effects of NAC on the BB-induced release of LDH and ALT

The release of LDH and ALT into the medium from control slices and slices incubated with 10 mM NAC alone was < 35% and < 15% of total slice activity after 6 h in culture respectively (Figure 8.2). Approximately 4- and 2-fold increases of LDH and ALT leakage were observed after exposure to 1 mM BB. This increased leakage was subsequently reduced by inclusion of NAC in a concentration-dependent manner (Figure 8.2).

8.2.3 Effects of NAC on the BB-induced TBARS Formation

The release of TBARS into the incubation medium from control slices was approximately 20 nmol/100 mg protein after 6 h in culture. Addition of 10 mM NAC to the culture medium did not significantly change the formation of TBARS by slices. BB exposure produced a time-dependent increase in TBARS formation such that it had increased by approximately 5-fold after 6 h incubation. This was dramatically inhibited by the inclusion of NAC in the culture medium. Indeed, a concentration dependency was not seen clearly in that NAC at 1 mM or above completely restored TBARS formation to values similar to those for control slices (Figure 8.3).

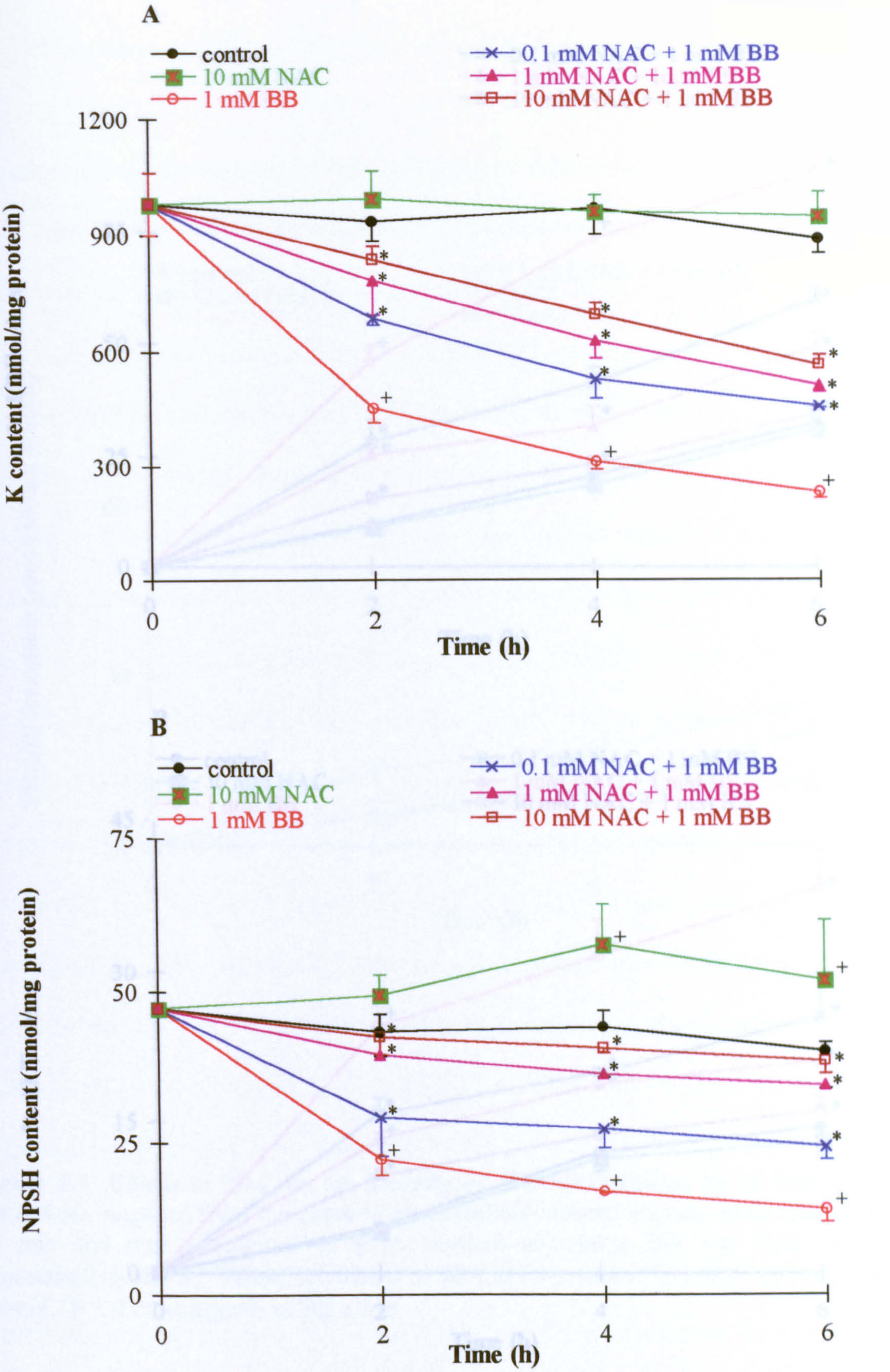


Figure 8.1 Effects of NAC on the BB-induced loss of K^+ (A) and depletion of NPSH (B) from rat liver slices. Slices were prepared from the livers of phenobarbital-induced animals, preincubated for 30 min and then transferred to fresh medium containing BB and NAC. Values are means ± SD from 3 animals. $^+P < 0.05$ compared to control, $^*P < 0.05$ compared to BB alone.

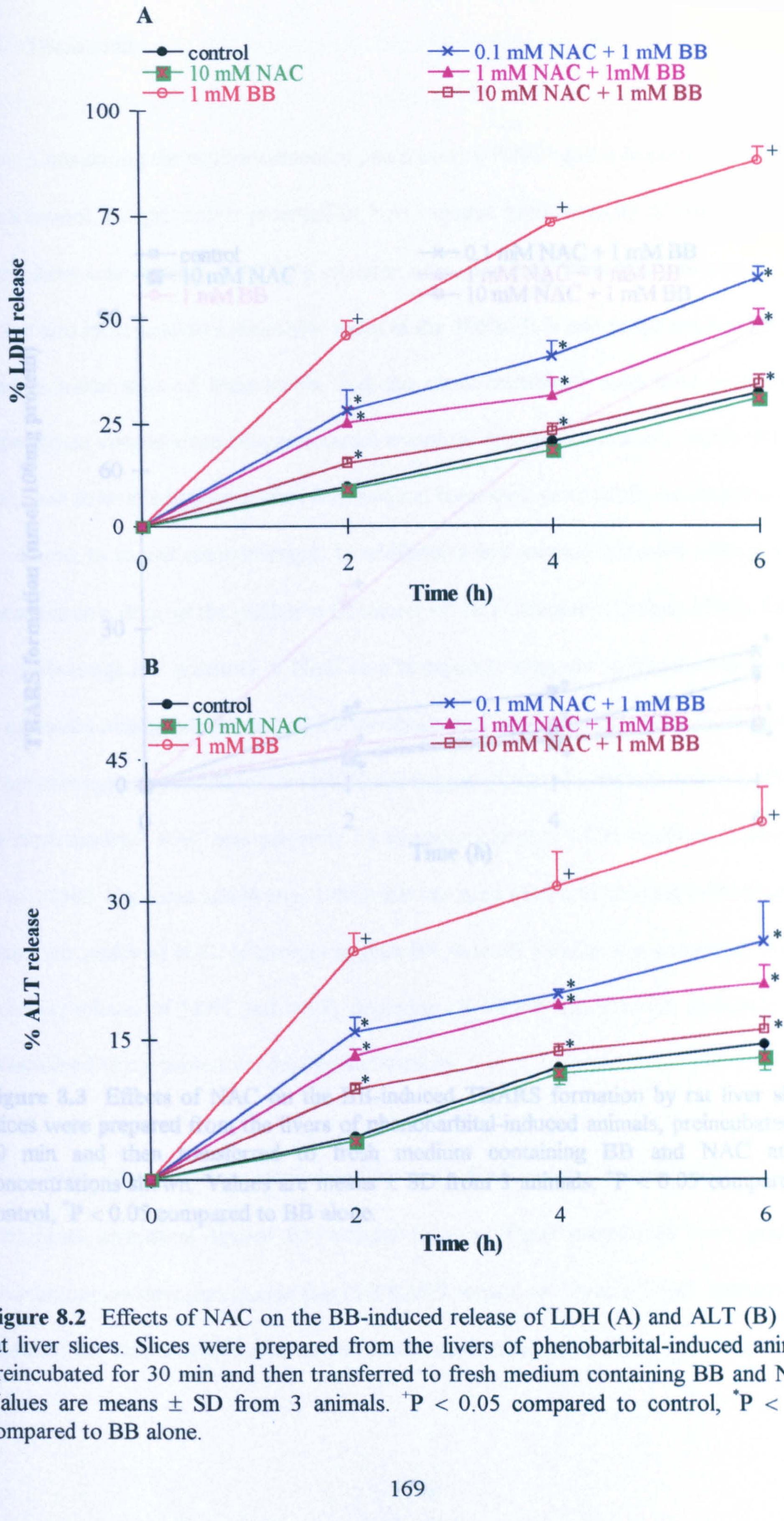


Figure 8.2 Effects of NAC on the BB-induced release of LDH (A) and ALT (B) from rat liver slices. Slices were prepared from the livers of phenobarbital-induced animals, preincubated for 30 min and then transferred to fresh medium containing BB and NAC. Values are means ± SD from 3 animals. $^{+}P < 0.05$ compared to control, $^{*}P < 0.05$ compared to BB alone.

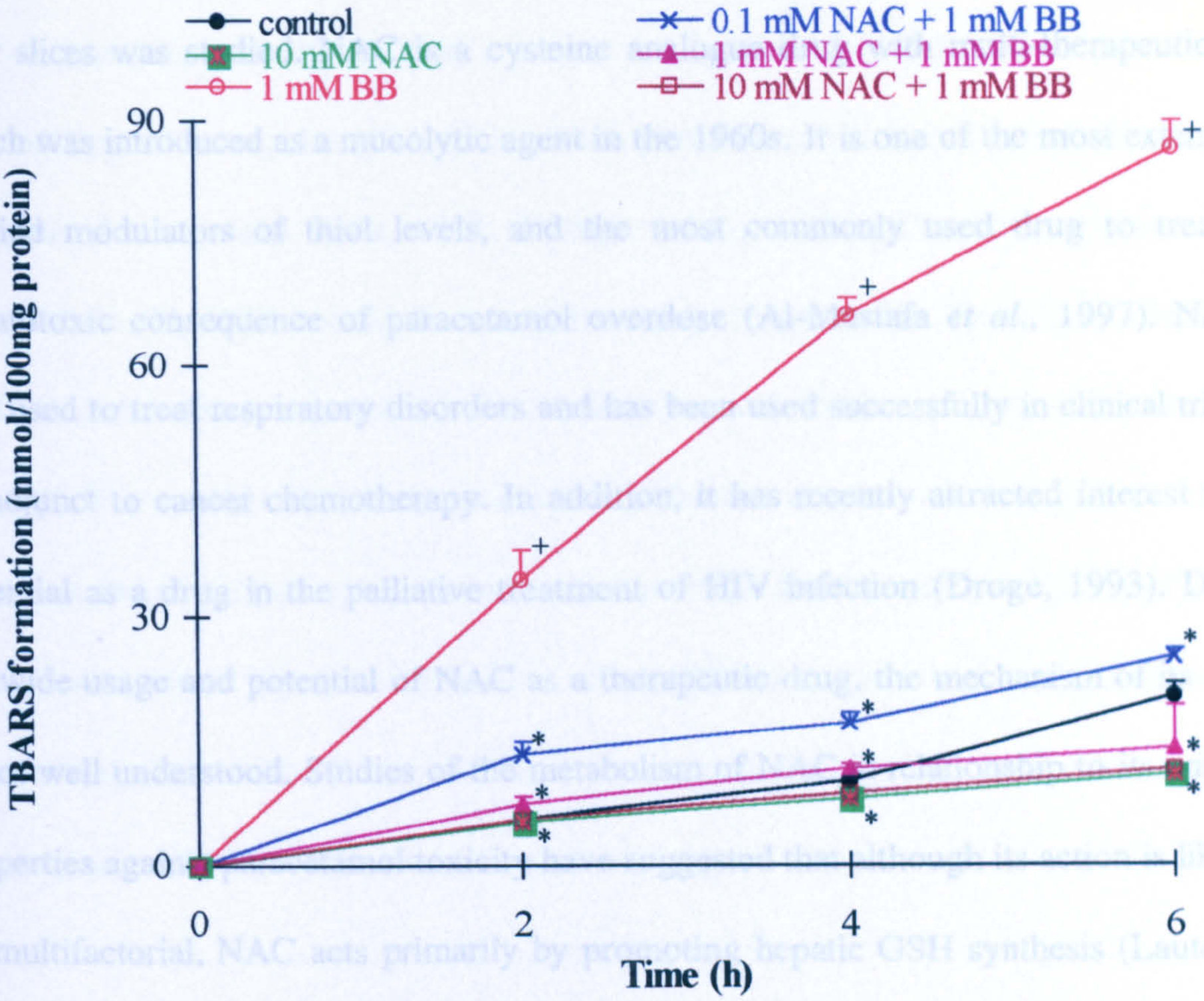


Figure 8.3 Effects of NAC on the BB-induced TBARS formation by rat liver slices. Slices were prepared from the livers of phenobarbital-induced animals, preincubated for 30 min and then transferred to fresh medium containing BB and NAC at the concentrations shown. Values are means ± SD from 3 animals. $^{+}P < 0.05$ compared to control, $^{*}P < 0.05$ compared to BB alone.

8.3 Discussion

Considering the well-documented protection by NAC against hepatotoxicity due to paracetamol, the preventive potential of NAC against hepatotoxicity of BB towards rat liver slices was studied. NAC is a cysteine analogue drug with multi-therapeutic uses which was introduced as a mucolytic agent in the 1960s. It is one of the most extensively studied modulators of thiol levels, and the most commonly used drug to treat the hepatotoxic consequence of paracetamol overdose (Al-Mustafa *et al.*, 1997). NAC is also used to treat respiratory disorders and has been used successfully in clinical trials as an adjunct to cancer chemotherapy. In addition, it has recently attracted interest for its potential as a drug in the palliative treatment of HIV infection (Droge, 1993). Despite the wide usage and potential of NAC as a therapeutic drug, the mechanism of its action is not well understood. Studies of the metabolism of NAC in relationship to its antidotal properties against paracetamol toxicity have suggested that although its action is likely to be multifactorial, NAC acts primarily by promoting hepatic GSH synthesis (Lauterburg *et al.*, 1983; Corcoran and Wong, 1986). BB has been shown to produce GSH depletion. Thus, the ability of NAC to protect against BB-induced toxicity was examined. Slice K^+ content, release of LDH and ALT, depletion of NPSH and TBARS formation were determined as parameters of viability and toxicity.

With all parameters studied, addition of NAC to the culture medium offered significant protection against BB-induced toxicity. These protections were time- and concentration-dependent except that of TBARS formation. Thus, TBARS formation was reduced to control levels even at the lower concentration of NAC and hence a clear

concentration dependency was not observed. The decreases in the loss of slice K^+ and release of LDH and ALT caused by BB indicate that NAC protects against the loss of hepatocyte membrane integrity.

It has been established that a major mechanism of prevention of paracetamol-induced hepatotoxicity by NAC is its deacetylation to cysteine, a precursor for GSH synthesis (Massey and Racz, 1981; Rafeiro *et al.*, 1994). The reactive paracetamol intermediate (NAPQI) would then bind to the increased GSH. Therefore, addition of NAC to liver slices exposed to BB should prevent GSH depletion and subsequent toxicity. This would support the concept that the reactive metabolite and not the high concentration of the parent compound was responsible for the cytotoxicity. These finding agreed with those reported by Jollow *et al.* (1974), in which the administration of cysteine *in vivo* restored the NPSH content depleted by BB, and prevented any histological evidence of BB-induced hepatotoxicity.

NAC was found to markedly reduce lipid peroxidation and the decline in hepatic NPSH content caused by BB. Others have demonstrated a similar protection of NAC against BB-induced toxicity (Thor *et al.*, 1979; Brodeur and Goyal, 1987; Grewal *et al.*, 1996). NAC has been found to protect the liver against an injury by several xenobiotics such as carbon tetrachloride and cocaine through its potential to restore tissue GSH, promoting the conjugation and detoxification of reactive metabolites (Suarez *et al.*, 1986; Simko *et al.*, 1992). Since NAC has been reported to promote GSH synthesis in the mouse following paracetamol challenge it would be reasonable to speculate that NAC in the present studies protects against BB hepatotoxicity by a similar mechanism.

Several authors have reported previously that under certain conditions NAC can increase hepatic GSH levels in mice by up to three times that of control values (Corcoran and Wong, 1986; Harman and Self, 1986; Wong *et al.*, 1986; Al-Mustafa *et al.*, 1997). In this study, the NPSH (mainly GSH) content of rat liver slices did not increase substantially following the addition of NAC alone to the culture medium. Similar results have been obtained by Hayes *et al.* (1986), Yao *et al.* (1994) and McLellan *et al.* (1995). These data suggest perhaps that NAC only stimulates GSH synthesis in GSH-depleted tissue.

In summary, the results from these studies demonstrate that NAC is an effective antidote against BB-induced hepatotoxicity and this may be attributed primarily to its direct or indirect (via cysteine or GSH) nucleophilic capacity to conjugate and detoxify the reactive metabolites derived from BB.

CHAPTER 9

EFFECTS OF BUTHIONINE SULPHOXIMINE ON PROTECTION AGAINST BROMOBENZENE TOXICITY BY AGED GARLIC EXTRACT, S-ALLYL CYSTEINE AND N-ACETYL CYSTEINE

It is generally accepted that GSH has an important function in chemical detoxification processes. BB hepatotoxicity is known to occur only after cellular stores of GSH have been depleted. BSO is a specific and irreversible inhibitor of γ-glutamylcysteine synthetase (the rate limiting enzyme in GSH synthesis, see Section 1.2.2) and hence blocks GSH synthesis. This leads to a depletion of cellular GSH pools by the ongoing reactions of GSH utilisation (Jurima-Romet *et al.*, 1996). It has been reported that the GSH content in isolated mouse hepatocytes was significantly decreased after incubation for 1.5 h with 1 mM BSO and this reduction was maintained for a further 4 h of incubation (Rafeiro *et al.*, 1994). It has been also demonstrated that 1 mM BSO is sufficient to deplete cellular GSH without any loss of cell viability. Thus, in this study 1 mM BSO was added to the incubation medium of rat liver slices in order to inhibit GSH synthesis and study the effect of this on BB toxicity and its prevention by AGE, SAC and NAC.

9.1 Methods

Animals were pretreated with sodium phenobarbital as described in Section 2.2. All slices used in these experiments were prepared from the livers of phenobarbital-induced rats and cultured for up to 6 h as described in Sections 2.3 - 2.6.

BB solutions were prepared in DMSO and added to the culture medium such that the final DMSO concentration was 1% (v/v). AGE was added directly to the culture medium to achieve the final desired concentration. SAC , NAC and BSO were dissolved in dH_2O to a stock concentration of 0.1 M, filtered with disposable syringe filters and stored at - 70 °C. BSO was added to the culture medium either at the same time as the other test compounds or 2 h prior to the addition of the other test compounds at the indicated concentrations. All slices were preincubated in DMEM for 30 min at 37 °C before any experiments were undertaken.

The effects of BSO on the protective effects of AGE, SAC and NAC towards BB-induced depletion of GSH, loss of K^+ and release of LDH were determined and expressed as described in Section 2.8.

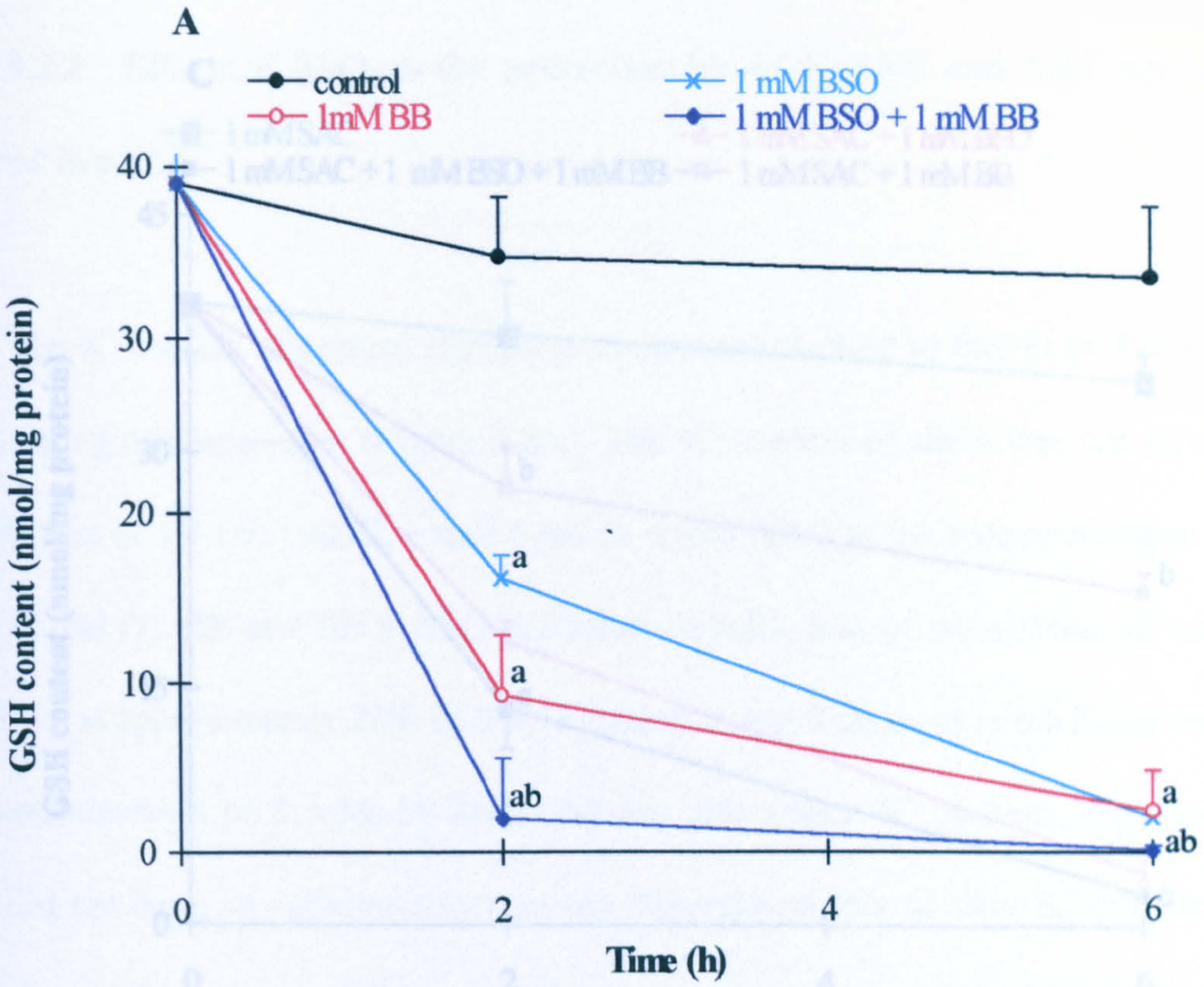
9.2 Results

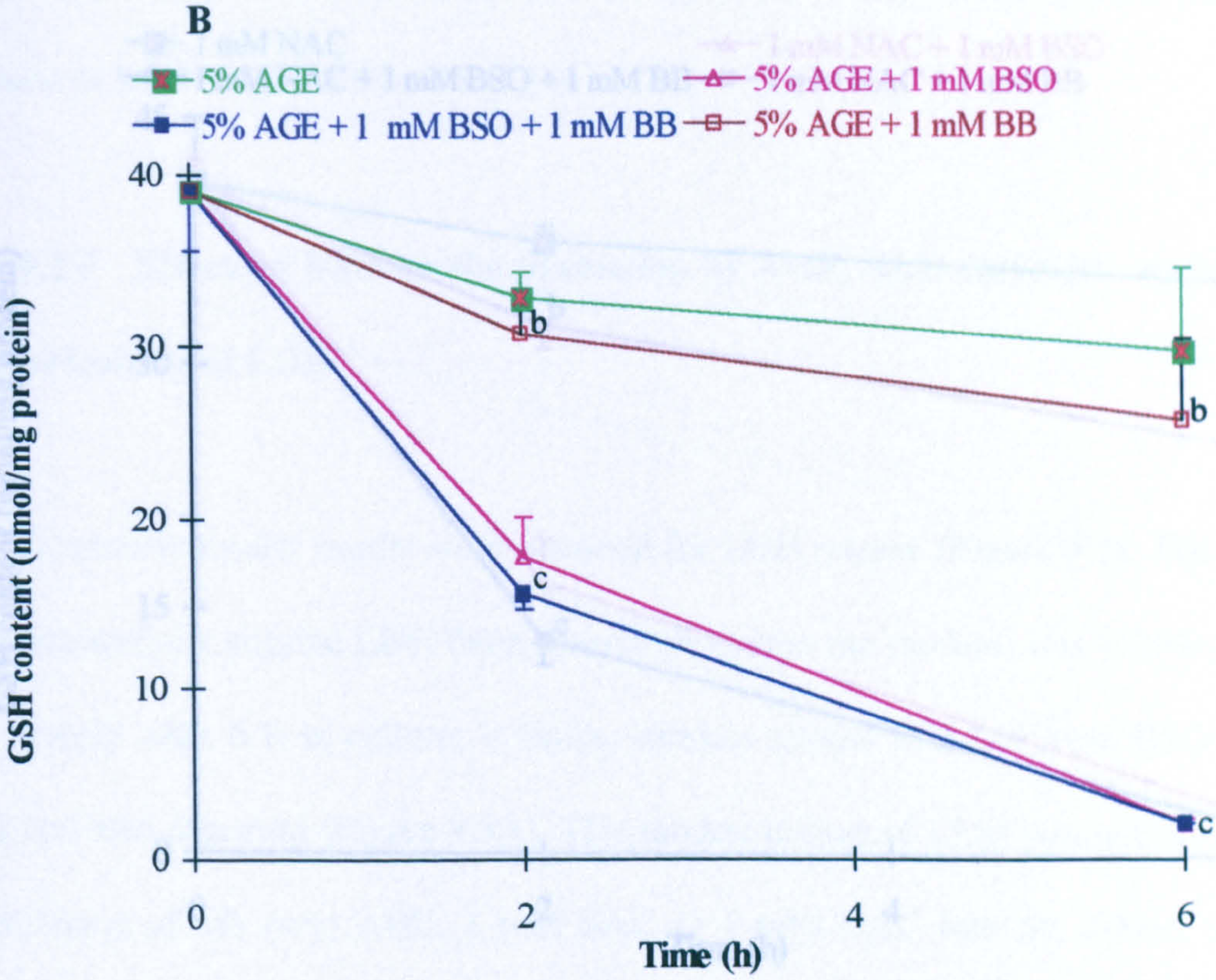
9.2.1 Effects of BSO on the protection by AGE, SAC and NAC against BB-induced GSH depletion

The GSH content of control slices declined by only 15% of that in freshly-isolated slices during 6 h in culture (Figure 9.1A). GSH content of slices treated with AGE, SAC or NAC alone was not different from that of control slices (Figure 9.1B, C and D). A significant depletion in the total GSH content of slices incubated with BSO alone, BB alone and BB + BSO occurred within 2 h and progressed until there almost total depletion at 6 h. The GSH content of slices treated with BSO alone, BB alone and BB + BSO was approximately 45%, 25% and 5% of that of control slices by 2 h, respectively; thus, it is clear that the effects of BSO and BB are additive (Figure 9.1A).

The addition of 5% (v/v) AGE to BB-treated slices almost totally prevented the depletion of GSH whilst AGE did not reverse the depletion of GSH caused by BSO alone (Figure 9.1B). As shown in Figure 9.1B, AGE partially prevented the depletion of GSH in BB-treated slices in the presence of BSO. However, this protection was not as marked as that observed in BB-treated slices in the absence of BSO. Similar results were observed upon inclusion of 1 mM SAC and 1 mM NAC in the culture medium as shown in Figure 9.1C and D.

Effects of BSO on protection against BB toxicity by AGE, SAC and NAC





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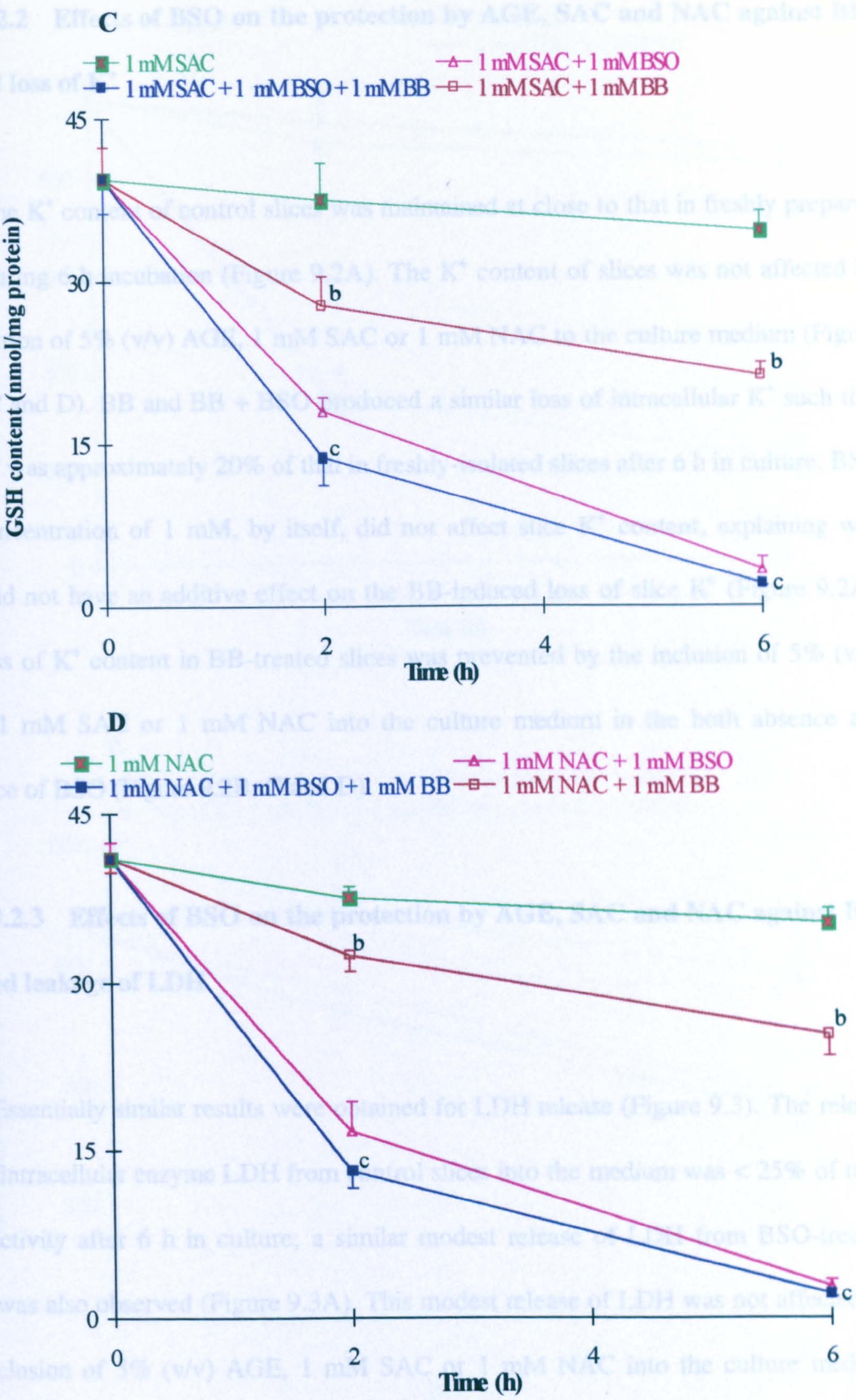


Figure 9.1 Effects of BSO on the protection by AGE (B), SAC (C) and NAC (D) of BB-induced GSH depletion in rat liver slices. AGE (or SAC, NAC), BB and BSO were added to the incubation medium at the same time. Values are means ± SD from at least 3 animals. $^{a}P < 0.05$ compared to control, $^{b}P < 0.05$ compared to BB and $^{c}P < 0.05$ compared to BSO plus BB.

9.2.2 Effects of BSO on the protection by AGE, SAC and NAC against BB-induced loss of K^+

The K^+ content of control slices was maintained at close to that in freshly prepared slices during 6 h incubation (Figure 9.2A). The K^+ content of slices was not affected by the addition of 5% (v/v) AGE, 1 mM SAC or 1 mM NAC to the culture medium (Figure 9.2B, C and D). BB and BB + BSO produced a similar loss of intracellular K^+ such that slice K^+ was approximately 20% of that in freshly-isolated slices after 6 h in culture. BSO at a concentration of 1 mM, by itself, did not affect slice K^+ content, explaining why BSO did not have an additive effect on the BB-induced loss of slice K^+ (Figure 9.2A). The loss of K^+ content in BB-treated slices was prevented by the inclusion of 5% (v/v) AGE, 1 mM SAC or 1 mM NAC into the culture medium in the both absence and presence of BSO (Figure 9.2B, C and D).

9.2.3 Effects of BSO on the protection by AGE, SAC and NAC against BB-induced leakage of LDH

Essentially similar results were obtained for LDH release (Figure 9.3). The release of the intracellular enzyme LDH from control slices into the medium was < 25% of total slice activity after 6 h in culture; a similar modest release of LDH from BSO-treated slices was also observed (Figure 9.3A). This modest release of LDH was not affected by the inclusion of 5% (v/v) AGE, 1 mM SAC or 1 mM NAC into the culture medium (Figure 9.3B, C and D). BB at 1 mM increased the release of LDH by approximately 3-fold in the absence and presence of BSO. Thus, as with slice K^+ content, BSO did not

Effects of BSO on protection against BB toxicity by AGE, SAC and NAC

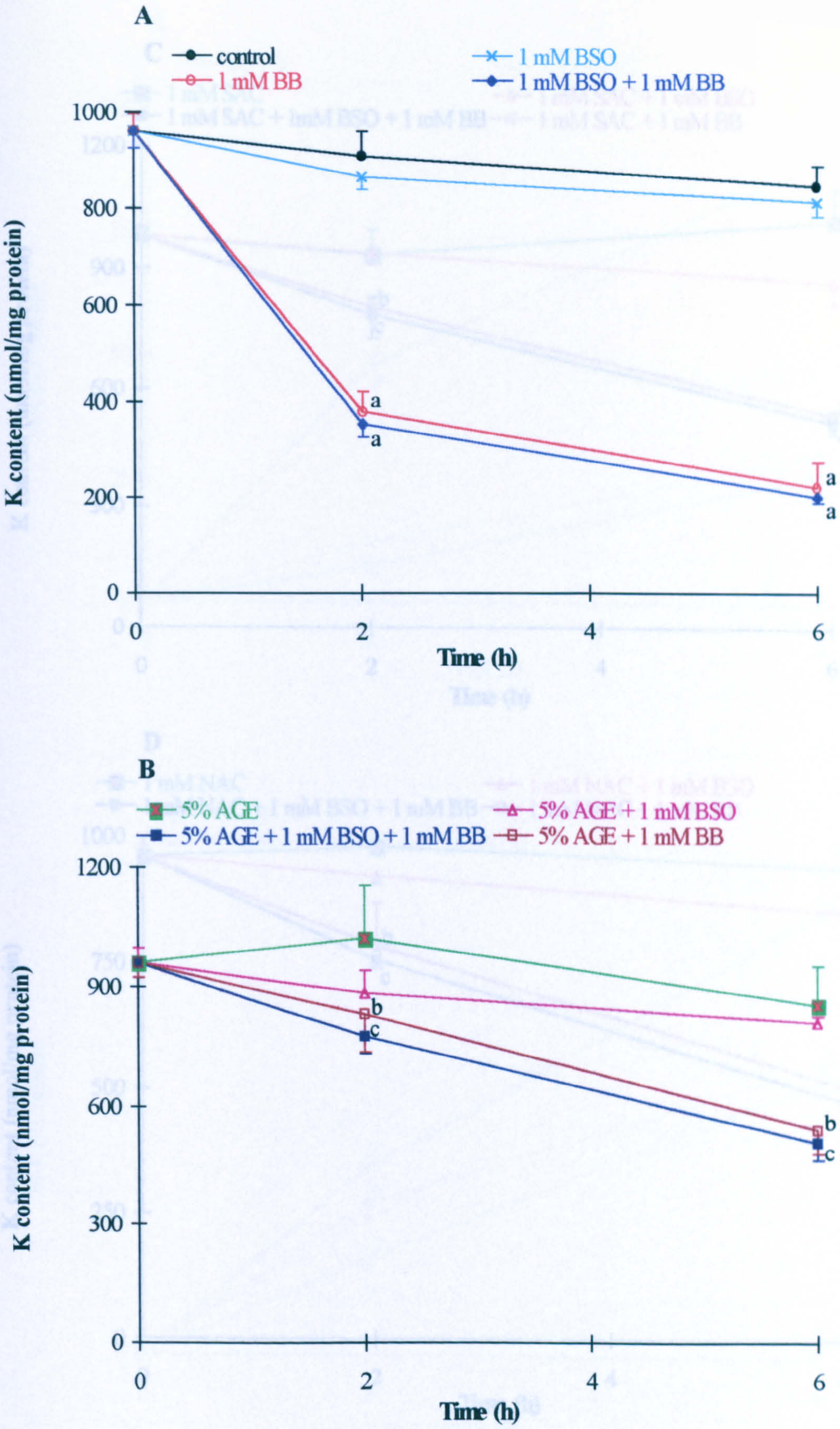


Figure 9.2 See over for legend.

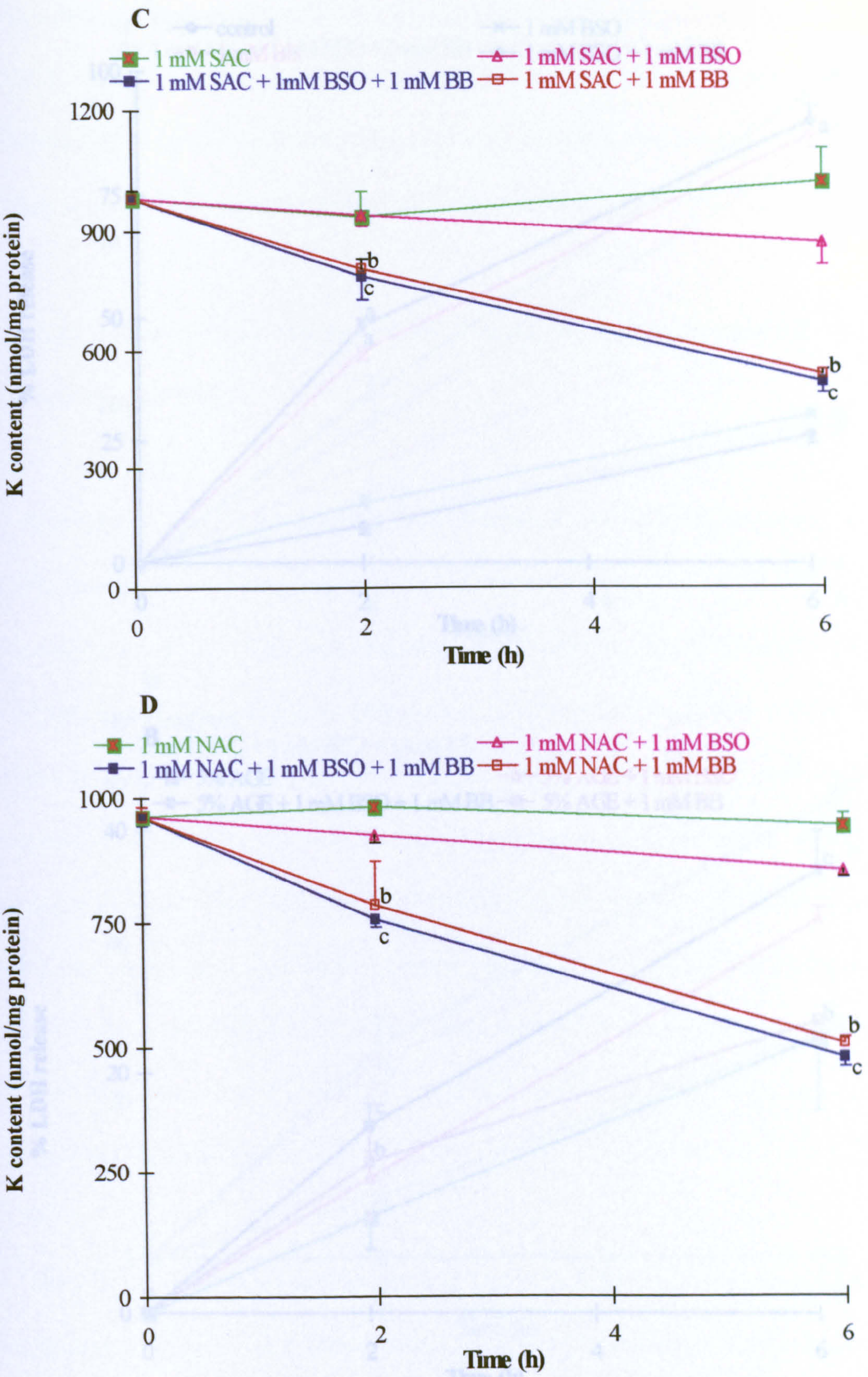
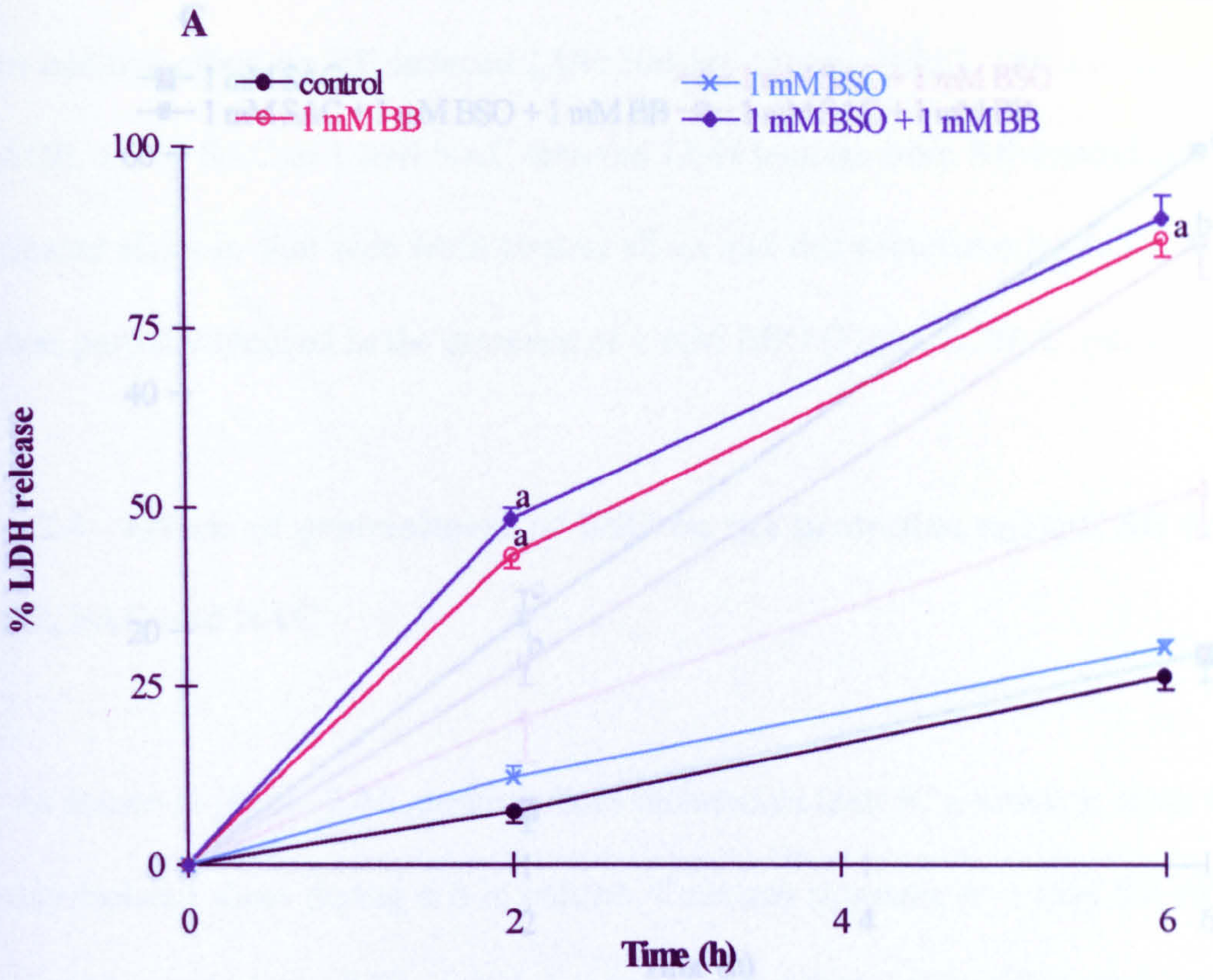
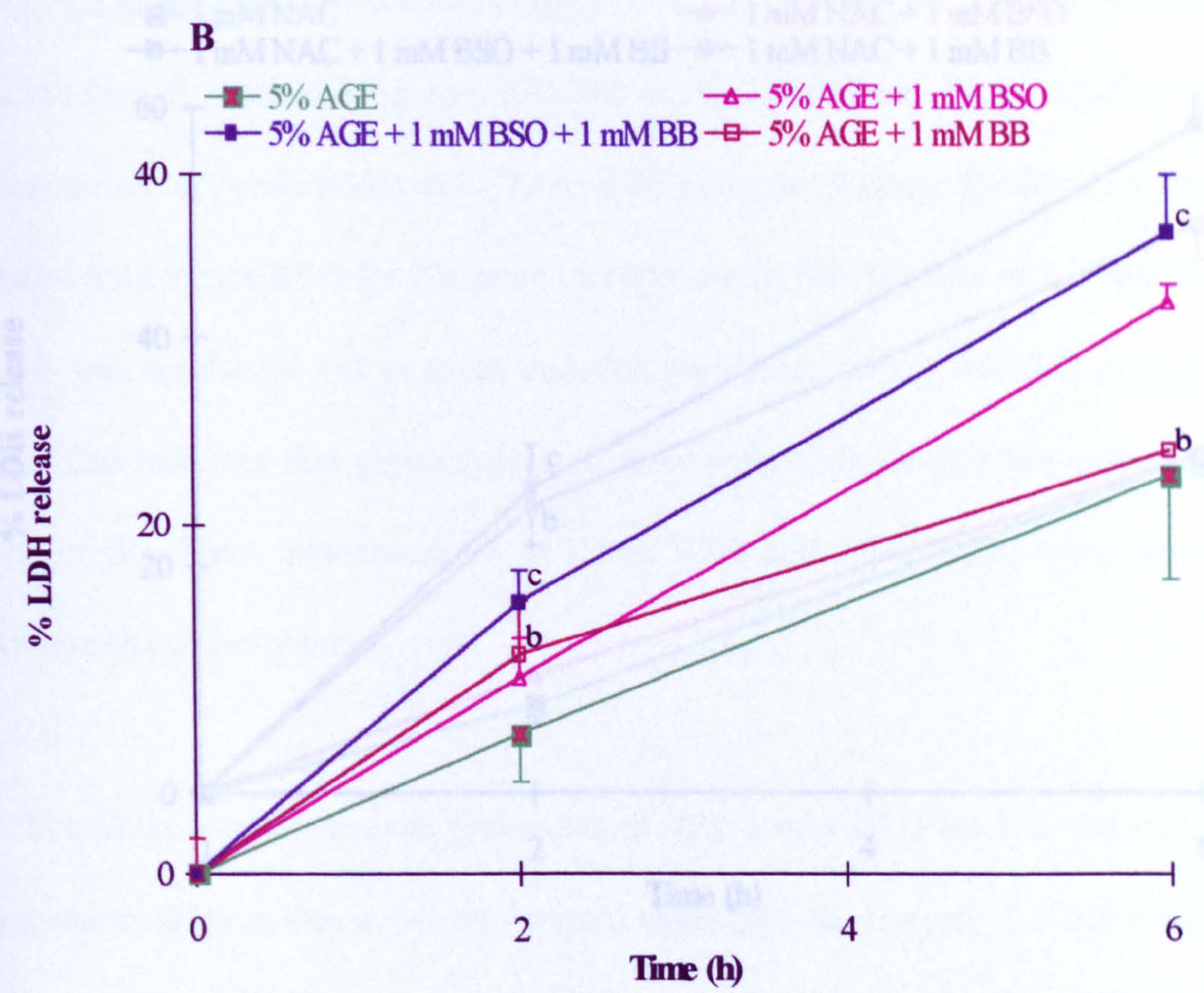


Figure 9.2 Effects of BSO on the protection by AGE (B), SAC (C) and NAC (D) of BB-induced loss of K^+ from rat liver slices. AGE (or SAC, NAC), BB and BSO were added to the incubation medium at the same time. Values are means ± SD from at least 3 animals. $^aP < 0.05$ compared to control, $^bP < 0.05$ compared to BB and $^cP < 0.05$ compared to BSO plus BB.

Effects of BSO on protection against BB toxicity by AGE, SAC and NAC





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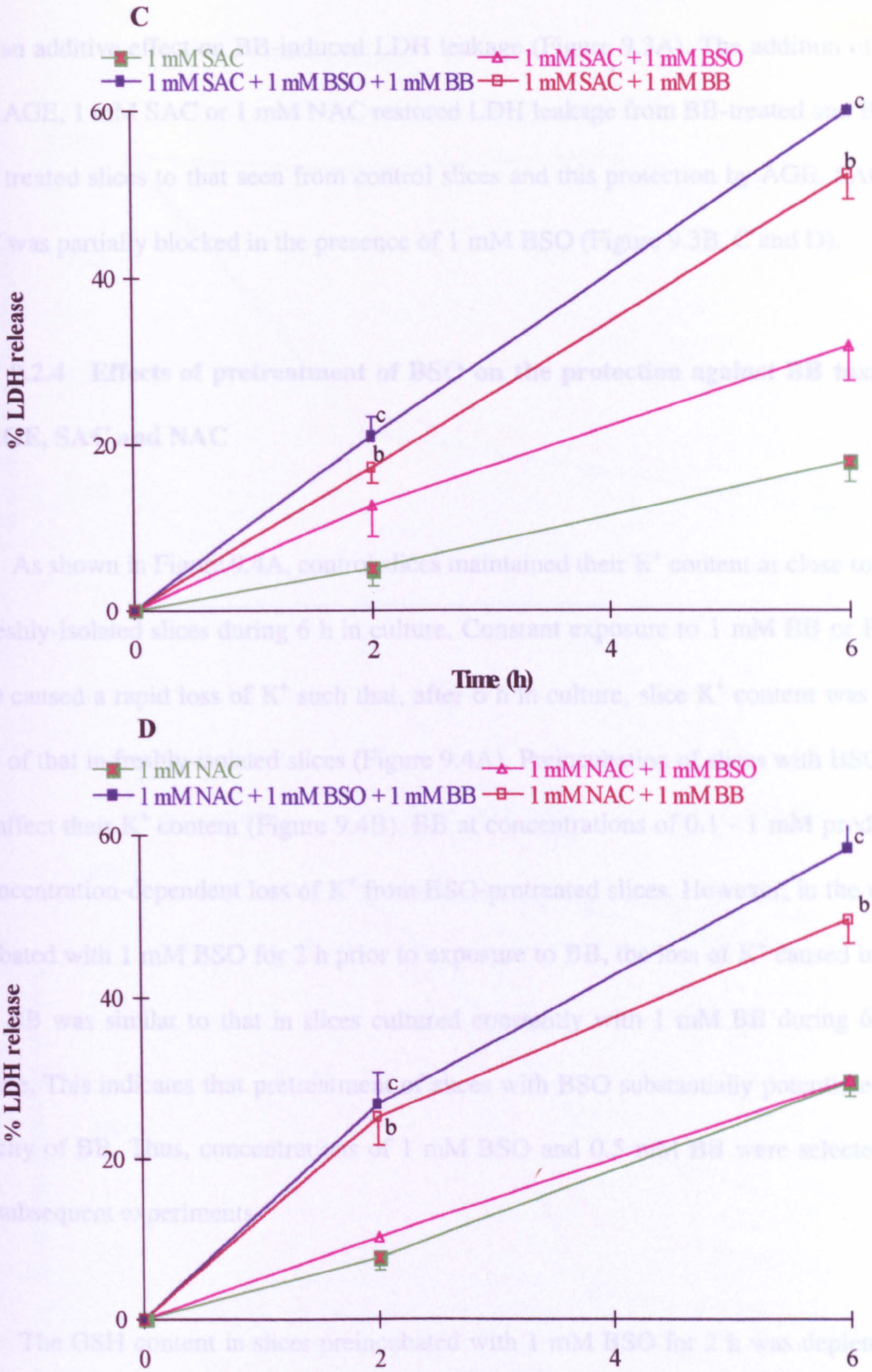


Figure 9.3 Effects of BSO on the protection by AGE (B), SAC (C) and NAC (D) of BB-induced leakage of LDH from rat liver slices. AGE (or SAC, NAC), BB and BSO were added to the incubation medium at the same time. Values are means ± SD from at least 3 animals. $^{a}P < 0.05$ compared to control, $^{b}P < 0.05$ compared to BB and $^{c}P < 0.05$ compared to BSO plus BB.

have an additive effect on BB-induced LDH leakage (Figure 9.3A). The addition of 5% (v/v) AGE, 1 mM SAC or 1 mM NAC restored LDH leakage from BB-treated and BB + BSO treated slices to that seen from control slices and this protection by AGE, SAC or NAC was partially blocked in the presence of 1 mM BSO (Figure 9.3B, C and D).

9.2.4 Effects of pretreatment of BSO on the protection against BB toxicity by AGE, SAC and NAC

As shown in Figure 9.4A, control slices maintained their K^+ content at close to that in freshly-isolated slices during 6 h in culture. Constant exposure to 1 mM BB or BB + BSO caused a rapid loss of K^+ such that, after 6 h in culture, slice K^+ content was only 25% of that in freshly-isolated slices (Figure 9.4A). Preincubation of slices with BSO did not affect their K^+ content (Figure 9.4B). BB at concentrations of 0.1 - 1 mM produced a concentration-dependent loss of K^+ from BSO-pretreated slices. However, in the slices incubated with 1 mM BSO for 2 h prior to exposure to BB, the loss of K^+ caused by 0.5 mM BB was similar to that in slices cultured constantly with 1 mM BB during 6 h in culture. This indicates that pretreatment of slices with BSO substantially potentiated the toxicity of BB. Thus, concentrations of 1 mM BSO and 0.5 mM BB were selected for the subsequent experiments.

The GSH content in slices preincubated with 1 mM BSO for 2 h was depleted by approximately 65% of that in freshly-isolated slices and no recovery of GSH content was observed over a 4 h incubation period in fresh medium (Figure 9.5). The GSH content of slices pretreated with BSO was further decreased to approximately 10% of that in freshly-isolated slices by the addition of 0.5 mM BB. This additional decrease in GSH

Effects of BSO on protection against BB toxicity by AGE, SAC and NAC

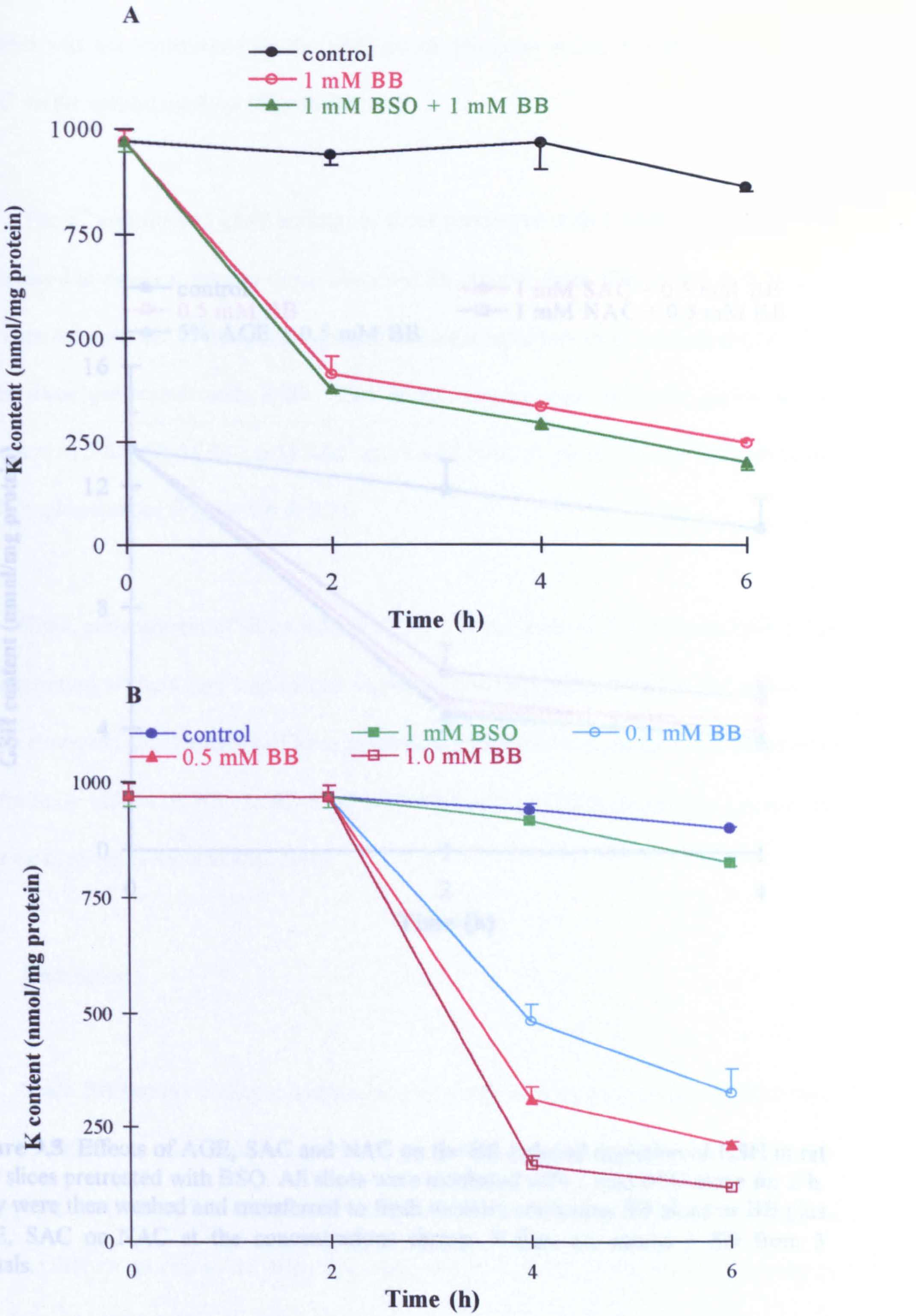


Figure 9.4 BB-induced loss of K^+ from rat liver slices. (A) Liver slices were incubated with BB or BB + BSO continually. (B) Liver slices were incubated for 2 h with 1 mM BSO alone; after transferring to fresh medium, BB or BSO was added at the concentrations shown.

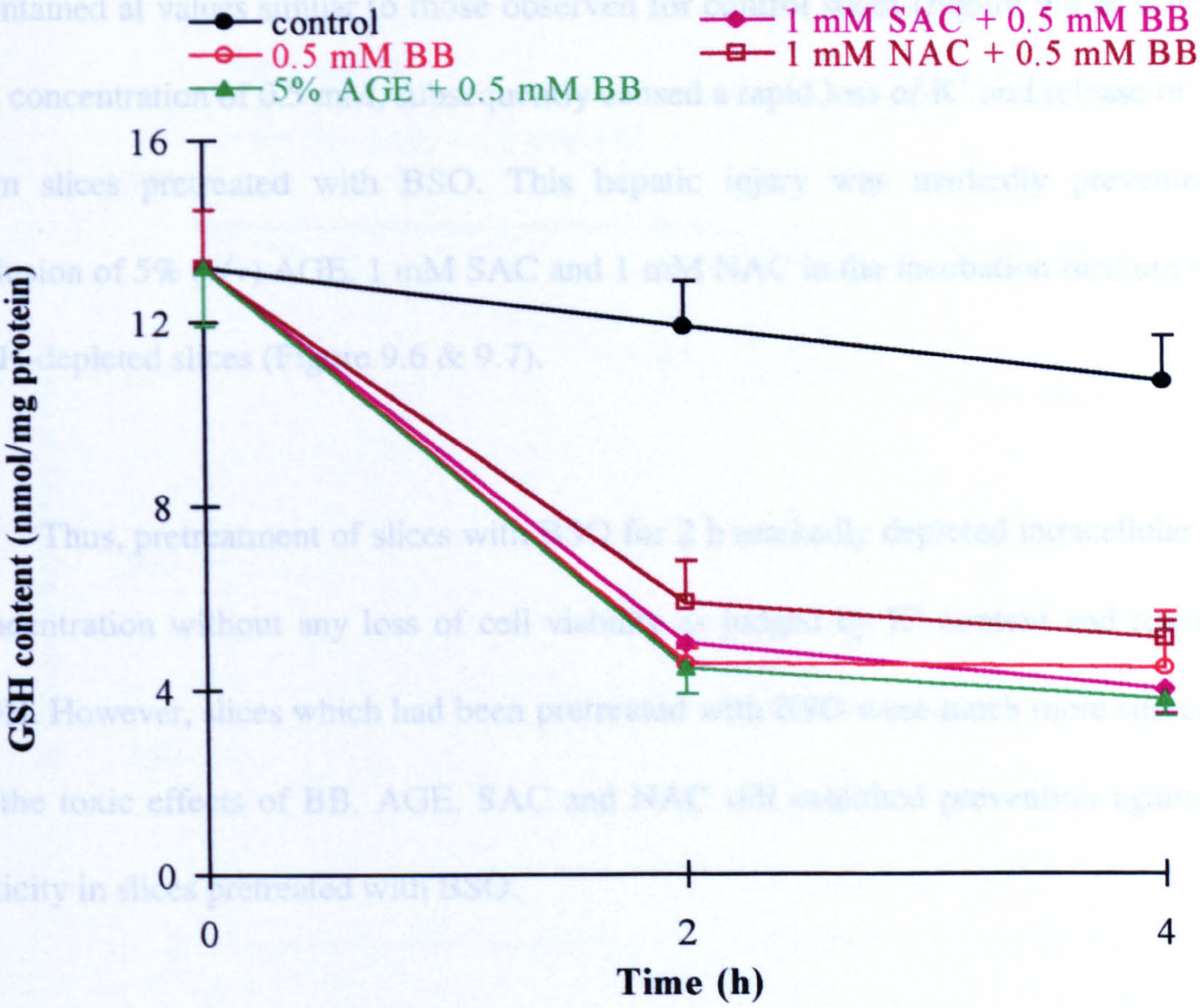


Figure 9.5 Effects of AGE, SAC and NAC on the BB-induced depletion of GSH in rat liver slices pretreated with BSO. All slices were incubated with 1 mM BSO alone for 2 h. They were then washed and transferred to fresh medium containing BB alone or BB plus AGE, SAC or NAC at the concentrations shown. Values are means ± SD from 3 animals.

content was not replenished by the addition of 5% (v/v) AGE, 1 mM SAC or 1 mM NAC to the culture medium (Figure 9.5).

The K^+ content and LDH leakage in slices pretreated with 1 mM BSO for 2 h was maintained at values similar to those observed for control slices (Figure 9.6 & 9.7). BB, at a concentration of 0.5 mM, subsequently caused a rapid loss of K^+ and release of LDH from slices pretreated with BSO. This hepatic injury was markedly prevented by inclusion of 5% (v/v) AGE, 1 mM SAC and 1 mM NAC in the incubation medium of the GSH-depleted slices (Figure 9.6 & 9.7).

Thus, pretreatment of slices with BSO for 2 h markedly depleted intracellular GSH concentration without any loss of cell viability as judged by K^+ content and release of LDH. However, slices which had been pretreated with BSO were much more susceptible to the toxic effects of BB. AGE, SAC and NAC still exhibited prevention against BB toxicity in slices pretreated with BSO.

9.3 Discussion

Since BB hepatotoxicity is known to occur only after cellular stores of GSH have been depleted, it has been hypothesised that the latent period before toxicity is manifested and the variation among animals were, at least in part, functions of varying hepatic GSH stores. Thus, the effects of AGE, SAC and NAC on BB-induced toxicity in the presence of BSO have been examined in order to investigate whether GSH synthesis is necessary for their protective effect. BSO is a potent and specific inhibitor of γ-

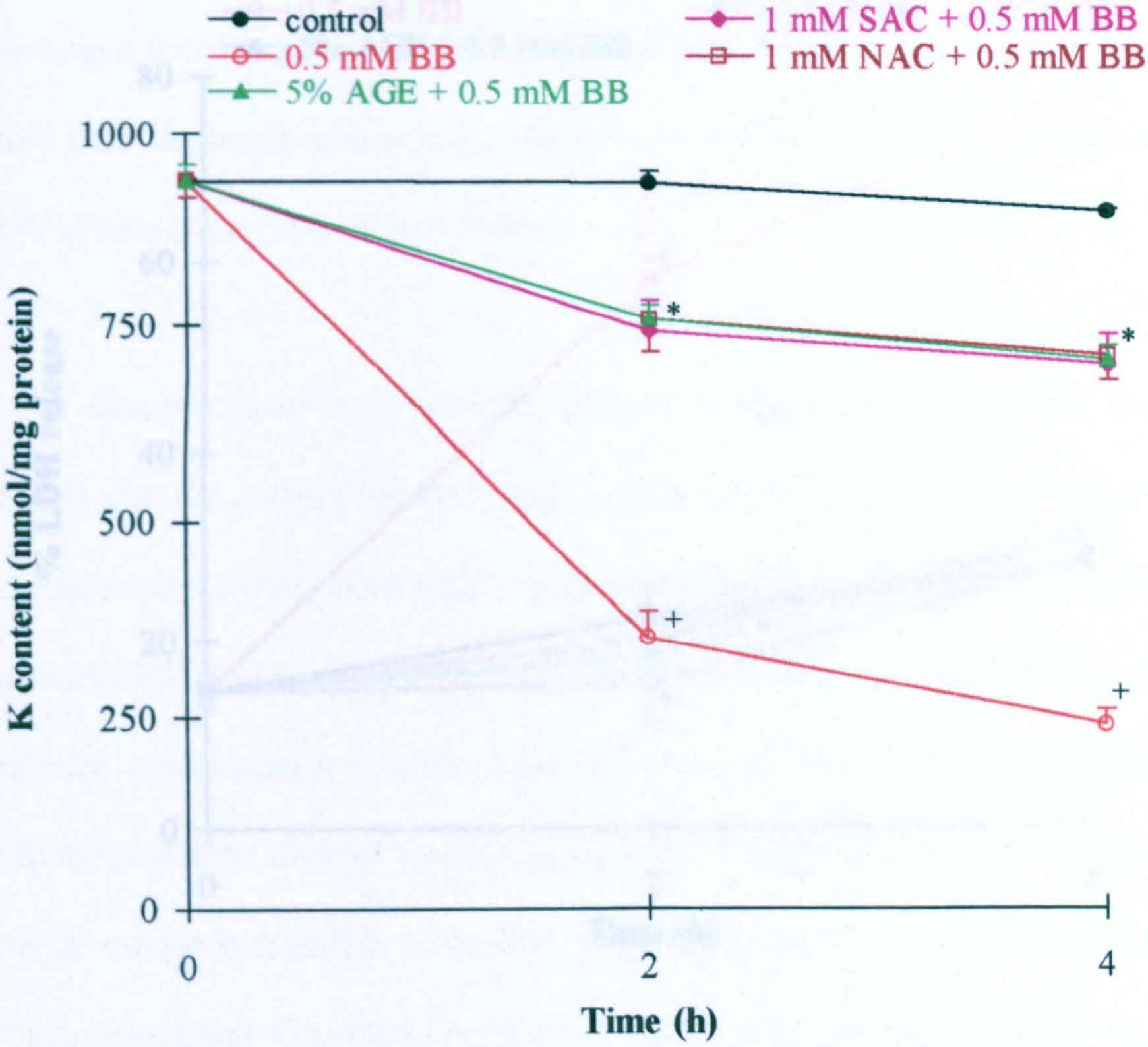


Figure 9.6 Effects of AGE, SAC and NAC on the BB-induced loss of K^+ from rat liver slices pretreated with BSO. All slices were incubated with 1 mM BSO alone for 2 h. They were then washed and transferred to fresh medium containing BB alone or BB plus AGE, SAC or NAC at the concentrations shown. Values are menas ± SD from 4 animals. $^{+}P < 0.05$ compared to control, $^{*}P < 0.05$ compared to BB alone.

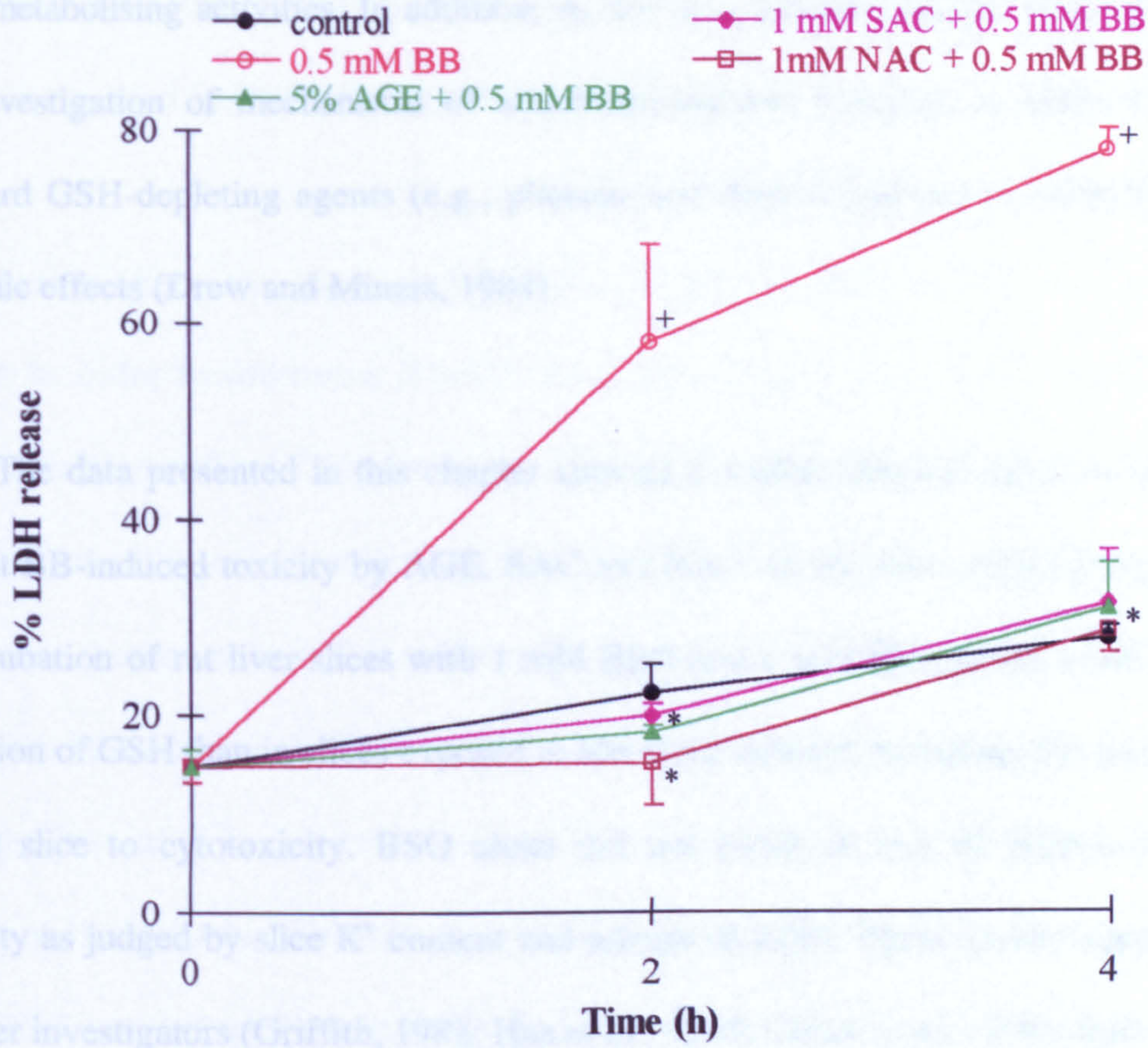


Figure 9.7 Effects of AGE, SAC and NAC on the BB-induced release of LDH from rat liver slices pretreated with BSO. All slices were incubated with 1 mM BSO alone for 2 h. They were then washed and transferred to fresh medium containing BB alone or BB plus AGE, SAC or NAC at the concentrations shown. Values are menas ± SD from 4 animals. $^{+}P < 0.05$ compared to control, $^{*}P < 0.05$ compared to BB alone.

glutamylcysteine synthetase (Griffith, 1982). This enzyme has a key role in the synthesis and maintenance of cellular GSH levels. It catalyses the conjugation of cysteine with glutamate to form γ-glutamylcysteine which then conjugates with glycine to form GSH. It has been reported that BSO does not affect cytochrome P-450 activities or phase II drug metabolising activities. In addition, its use as a specific depletor of tissue GSH in the investigation of mechanisms of xenobiotic-induced toxicities is preferable to the standard GSH-depleting agents (e.g., phorone and diethyl maleate) as these have other enzymic effects (Drew and Miners, 1984).

The data presented in this chapter showed a similar effect of BSO on protection against BB-induced toxicity by AGE, SAC and NAC. In the first series of experiments, co-incubation of rat liver slices with 1 mM BSO and 1 mM BB caused a much greater depletion of GSH than in slices exposed to BB alone without increasing the susceptibility of the slice to cytotoxicity. BSO alone did not result in loss of plasma membrane integrity as judged by slice K^+ content and release of LDH. These results support those of other investigators (Griffith, 1982; Hue *et al.*, 1985; Crook *et al.*, 1986; Rafeiro *et al.*, 1994; Hammond and Fry, 1996; Deas *et al.*, 1997) who demonstrated that BSO is an effective inhibitor of GSH synthesis without producing toxic effects *in vivo* and in cultured cells. The data shown in Chapter 8 showed that NAC increased NPSH content but did not raise GSH content suggesting that the addition of NAC to the incubation medium of slices does not lead to an increase in the level of GSH synthesis.

The protection against BB-induced toxicity exerted by AGE, SAC and NAC still occurred in BSO-treated slices. This agrees with the findings of others. For example,

NAC provides protection against enalapril-induced cytotoxicity in BSO-treated rat hepatocytes (Jurima-Romet *et al.*, 1991) and Offen *et al.* (1996) found that treatment with NAC rescued rat pheochromocytoma cells from the toxic effect of dopamine in the presence of BSO. These findings were confirmed by Nagasaki *et al.* (1998) who found that NAC prevented hepatic injury and improved liver integrity in livers in which GSH has been depleted by BSO after cold ischaemic-reperfusion injury.

In the experiments shown in Figure 9.1, 9.2 and 9.3 BSO was added with the other agents. In order to determine if AGE, SAC and NAC could exert a protective effect under conditions where GSH synthesis was already inhibited the effects of AGE, SAC and NAC on BB toxicity following the prior depletion of GSH by pretreatment with BSO were further examined in a second series of experiments. Pretreatment with BSO potentiated the toxicity of BB as judged by slice K^+ content such that 0.5 mM BB caused a similar loss of K^+ compared to exposure to 1 mM BB without BSO pretreatment (Figure 9.4). Similarly, Mizutani *et al.* (1994) reported an additive effect of pretreatment with BSO on dichlorobenzene-induced hepatotoxicity in mice. This potentiation of toxicity is not due to the intrinsic toxicity of BSO since treatment of slices with BSO alone did not result in any loss of cell viability.

The non-recovery of GSH synthesis after BSO removal is consistent with evidence that BSO is an irreversible inhibitor of γ-glutamylcysteine synthetase (Griffith, 1982). Addition of AGE, SAC or NAC to the culture medium did not raise the GSH content of slices following 2 h of BSO exposure whilst these agents still protected against hepatic injury caused by BB (Figures 9.5, 9.6 and 9.7). This indicates that the protection against

BB toxicity by AGE, SAC and NAC does not require GSH synthesis or rely on existing GSH in slices. The results presented here do not support those of Miners *et al.* (1984) who found pretreatment of mice with BSO abolished the protective effect of NAC on paracetamol-induced toxicity. Rafeiro *et al.* (1994) also demonstrated that NAC did not protect against paracetamol-induced cytotoxicity in cells in which GSH synthesis had been inhibited by BSO. These authors also reported that NAC was unable to replenish GSH content following depletion of GSH by BSO in paracetamol treated mouse hepatocytes. This is in agreement with the data presented in this study (Figure 9.5). It has been also reported that NAC prevented apoptosis in BSO-pretreated human peripheral T cells (Deas *et al.*, 1997) indicating that the protective effect of NAC was GSH-independent.

In conclusion, it has been demonstrated that AGE, SAC and NAC protected against BB-induced hepatotoxicity towards rat liver slices even when GSH was depleted by prior incubation with BSO. This suggests that these agents are capable of conjugating directly with reactive metabolites derived from BB rather than serving as substrates for GSH synthesis. NAC contains a free SH group, can react directly with electrophiles and is a potent free radical scavenger. These results suggest that SAC which is also a nucleophile can conjugate readily to BB-3,4-oxide.

CHAPTER 10

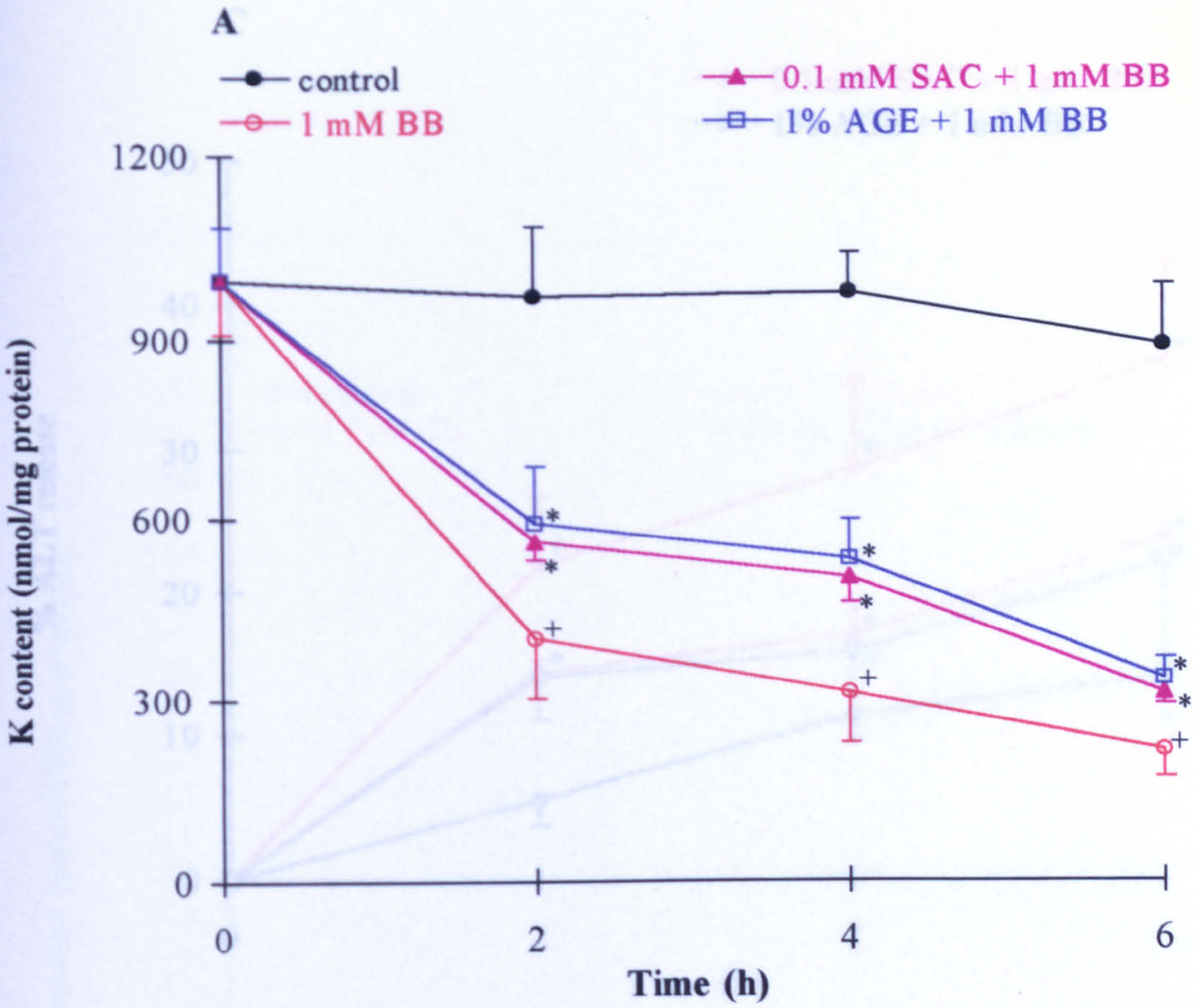
GENERAL DISCUSSION AND CONCLUSIONS

The functional and biochemical analysis of cultured liver slices has provided an integrated picture of the maintenance of these functions, which include membrane integrity, energy reserves, biosynthetic and secretory processes, and maintenance of drug metabolising enzymes. In most cases, these various processes were maintained at levels either equivalent to or better than those in primary hepatocytes cultures or other organ culture systems. Most functional indicators measured in this system were maintained equally. However, loss of P-450 activity occurred rapidly over the first 8 h of culture suggesting some common impairment in the system. However, this may be sufficient time to allow biotransformation of test compounds to occur, and thus the correct prediction of their *in vivo* toxicity. Using the classical hepatotoxin BB, the simple shaking organ culture method described here provided a system in which both descriptive and mechanistic hepatotoxicity studies were possible. The system permits the use of early, sensitive measures of cytotoxicity based on differentiated hepatocyte function to evaluate the potential toxicity of xenobiotics. Thus, it has become a reliable and affordable model for toxicological, pharmacological and metabolic studies.

The central aim of the work described in this thesis was to evaluate the effect of aged garlic extract (AGE) on the hepatotoxicity and metabolism of bromobenzene (BB). BB has been used as a model compound with which to investigate chemical-induced hepatotoxicity using cultured rat liver slices. The results presented in this thesis have shown that AGE added to the incubation medium of slices, or fed to rats for 7 days

before isolating slices, protects against the toxic effects of BB in a concentration (or dose) dependent manner. The hepatoprotective activities of S-allyl cysteine (SAC, the major organosulphur compound in AGE) and N-acetyl cysteine (NAC, another cysteine derivative) were shown to be similar to that of AGE. It has been shown that AGE protects against BB toxicity as a result of elevating hepatic GSH content (only seen after pretreating rats with AGE) and by functioning as a GSH-sparing agent, presumably due to conjugation of organosulphur compounds in AGE with the 3,4-oxide of BB. AGE and SAC have partial effect upon cytochrome P-450 activities and thus hepatoprotection cannot be primarily via inhibition of BB metabolism. The mechanism of prevention of BB toxicity by NAC could be due to its deacetylation to cysteine hence promoting GSH synthesis. However, in the present study, NAC alone did not substantially increase the GSH content of control slices suggesting that NAC may directly scavenge BB metabolites. Since GSH plays an important role in chemical detoxification processes, BSO was used as an inhibitor of GSH synthesis to investigate the mechanisms of BB toxicity. The results have shown that AGE, SAC and NAC also protect against BB toxicity towards liver slices in which GSH has been depleted by BSO. These findings further support the conclusion that the protective effects by AGE, SAC and NAC appear to be GSH-independent.

SAC is one of the most abundant organosulphur compounds in AGE. The batch of AGE used in this study contained 1.47 mg/ml SAC (i.e. 9.13 mM, see appendix 3). Therefore, a 1% (v/v) dilution of AGE is equivalent to 0.09 mM SAC and thus comparison of 1% (v/v) AGE and 0.1 mM SAC is valid. As shown in Figure 10.1, 0.1 mM SAC and 1% (v/v) AGE exert a similar degree of partial protection against BB-



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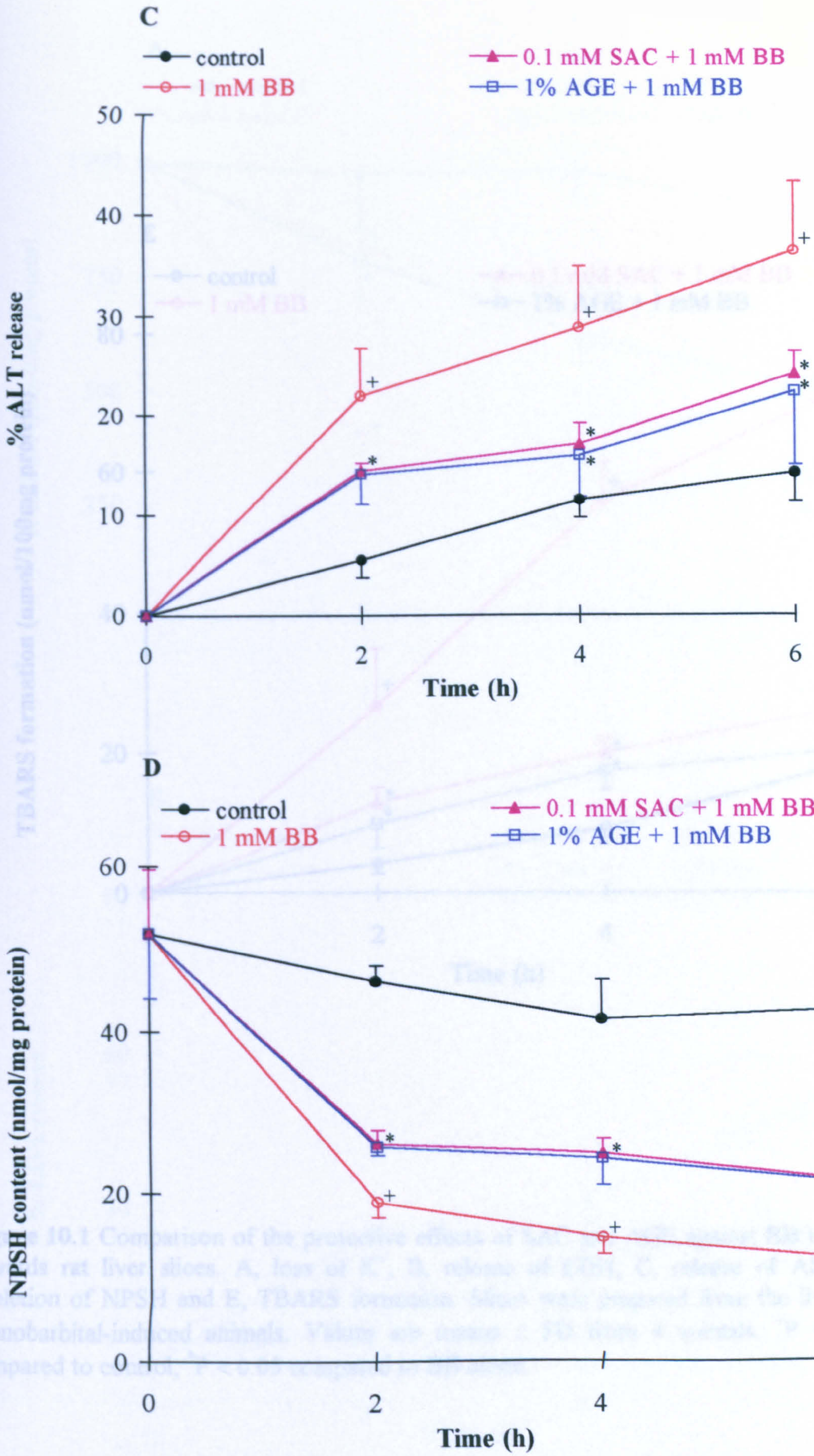

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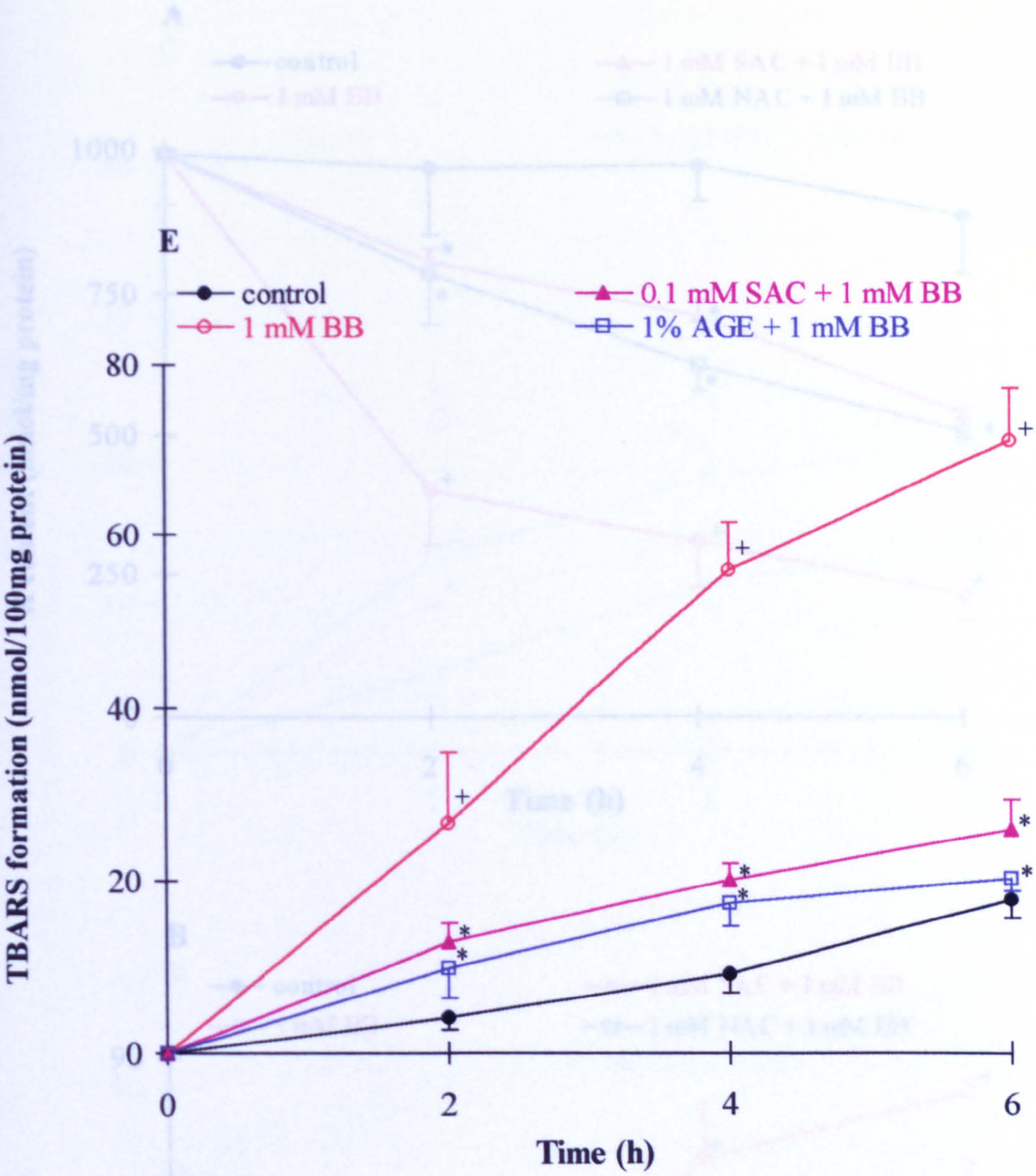
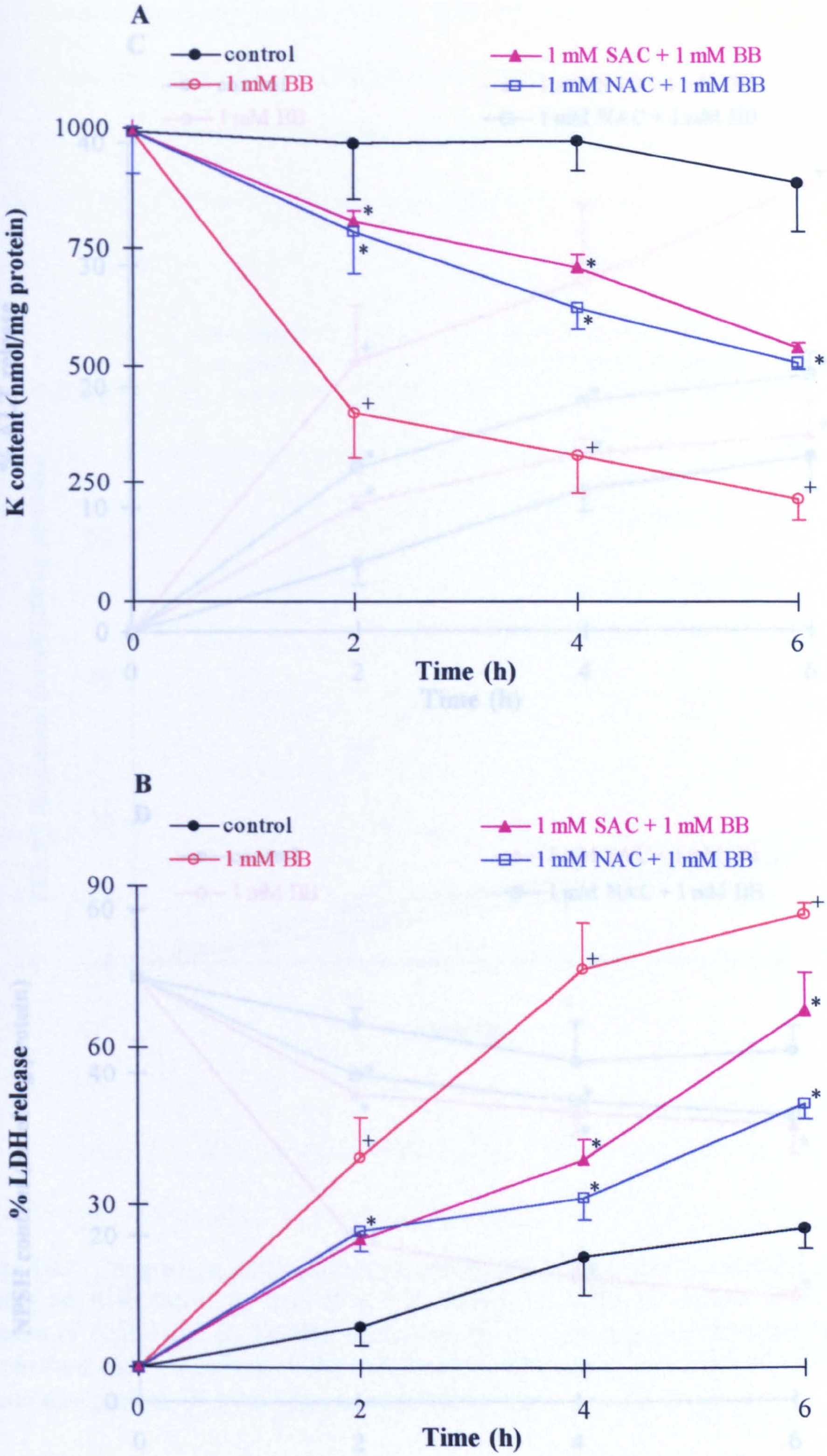


Figure 10.1 Comparison of the protective effects of SAC and AGE against BB toxicity towards rat liver slices. A, loss of K^+; B, release of LDH; C, release of ALT; D, depletion of NPSH and E, TBARS formation. Slices were prepared from the livers of phenobarbital-induced animals. Values are means ± SD from 4 animals. $^{+}P < 0.05$ compared to control, $^{*}P < 0.05$ compared to BB alone.



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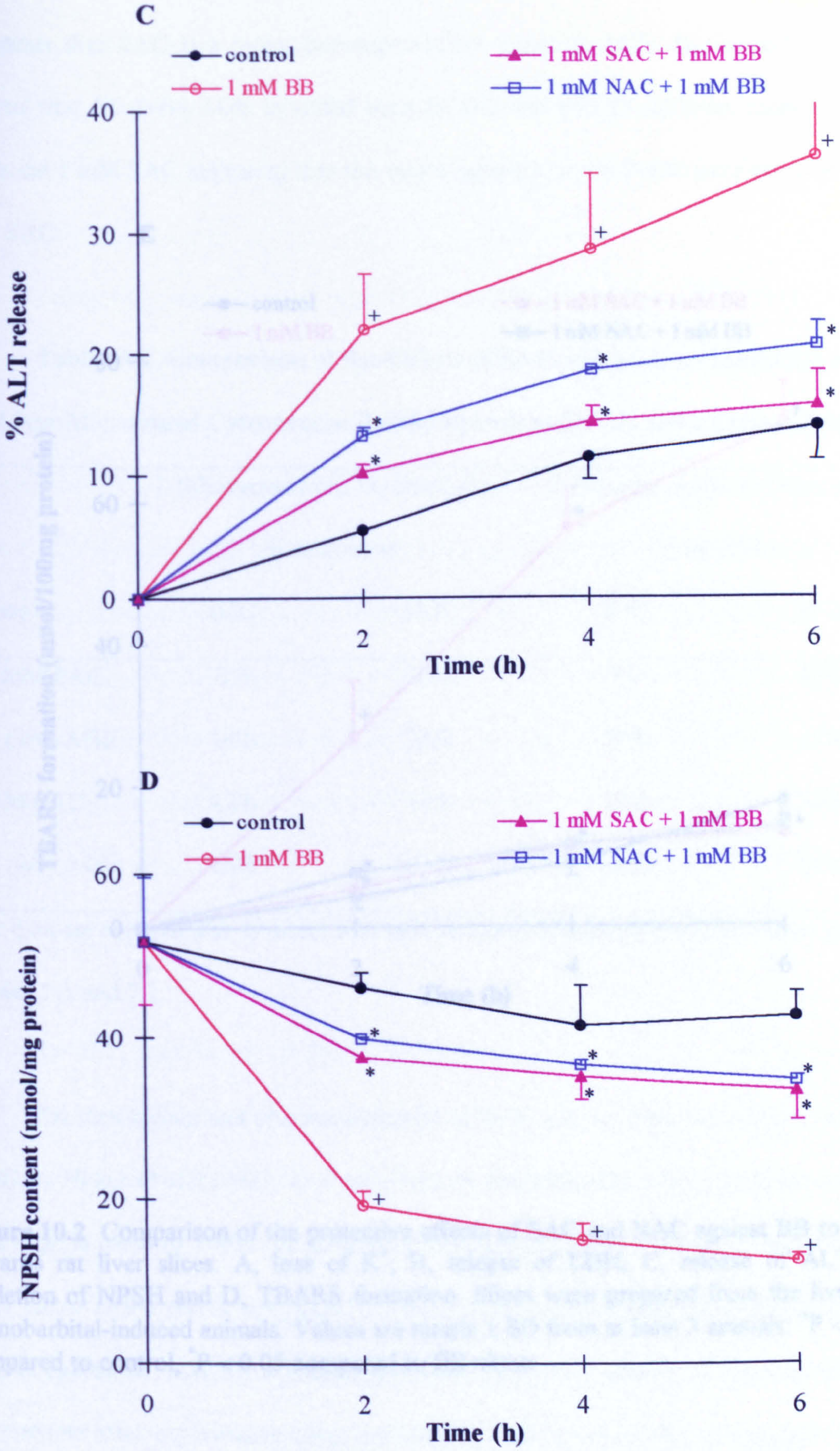

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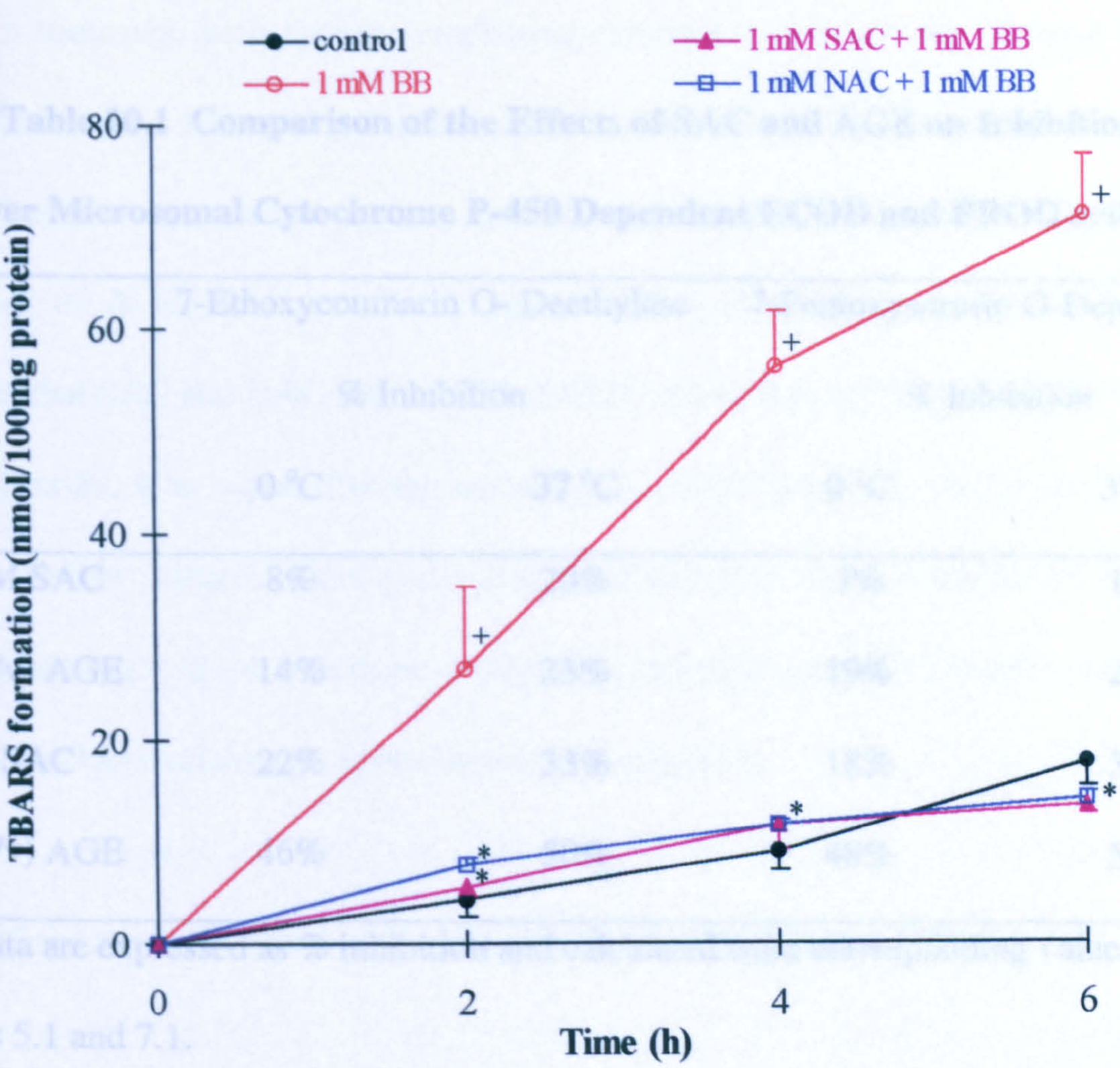

Figure 10.2 Comparison of the protective effects of SAC and NAC against BB toxicity towards rat liver slices. A, loss of K^+; B, release of LDH; C, release of ALT; D, depletion of NPSH and D, TBARS formation. Slices were prepared from the livers of phenobarbital-induced animals. Values are means ± SD from at least 3 animals. $^+P < 0.05$ compared to control, $^*P < 0.05$ compared to BB alone.

toxicity in rat liver slices as measured by five different parameters of viability. This suggests that SAC is a major hepatoprotective agent in AGE. However, Table 10.1 shows that 5% (v/v) AGE inhibited both ECOD and PROD activities more markedly than did 1 mM SAC indicating that the major inhibitor of the P-450 enzymes is probably not SAC.

Table 10.1 Comparison of the Effects of SAC and AGE on Inhibition of Rat Liver Microsomal Cytochrome P-450 Dependent ECOD and PROD activities

	7-Ethoxycoumarin O- Deethylase		7-Pentoxysorufin O-Depentylase	
	% Inhibition		% Inhibition	
Group	0 °C	37 °C	0 °C	37 °C
0.1 mM SAC	8%	20%	7%	19%
1% (v/v) AGE	14%	23%	19%	27%
1 mM SAC	22%	33%	18%	32%
5% (v/v) AGE	46%	50%	48%	57%

The data are expressed as % inhibition and calculated from corresponding values given in Tables 5.1 and 7.1.

The metabolism and pharmacokinetics of SAC are not fully understood. A single study by Nagae *et al.* (1994) has shown that the bioavailability of oral SAC in rats, mice and dogs is close to 100%. In addition, these authors showed that SAC is excreted in mice either as the parent compound or the N-acetyl form. If SAC or a metabolite is to protect against BB toxicity, a precise understanding of the mechanisms involved will only be possible after experiments using radiolabelled BB or SAC when conjugates may be

detected. It is possible that SAC acts as a nucleophile to trap directly the electrophilic metabolite BB-3,4-oxide. It is doubtful that SAC is hydrolysed to free cysteine; this was not detected by Nagae *et al.* (1994), nor did NPSH levels rise when slices were incubated with SAC (Figure 7.4).

In summary, such systems employing cultured liver slices can be used widely to evaluate both the toxic and protective effects of chemicals, and the cellular mechanisms by which toxicity and protection against toxicity result. Strongly hepatoprotective activities of AGE, SAC and NAC against BB toxicity were observed. The results indicate that SAC and NAC act as GSH-sparing agents through their ability to react with BB-3,4-oxide. It is hoped that the encouraging results from this preliminary study will lead to further research about garlic and eventually lead to the introduction of a cheap natural substance in the treatment of hepatic injury since garlic is a readily available and natural product containing various bioactive compounds.

Many avenues of further work are possible and include the following:

(1) The mechanisms of BB toxicity in rat hepatocytes are well understood, involving metabolic activation by cytochrome P-450 to form BB-3,4-oxide, an electrophilic molecule capable of binding covalently with tissue macromolecules, detoxification by epoxide hydrase and GSH transferase and the depletion of GSH (Jollow *et al.*, 1974). Some of the protective effect of AGE and SAC against BB toxicity might be due to modulations of phase I cytochrome P-450 enzymes and phase II detoxification enzymes such as glutathione S-transferase (GST) and epoxide hydrase (EH). The results

presented in this thesis showed partial inhibition of cytochrome P-450 activities *in vitro* by AGE and SAC and no effect *in vivo* by AGE when assayed as both 7-ethoxycoumarin O-deethylase and 7-pentoxyresorufin O-depentylase activities. Thus, the effects of AGE and/or SAC on other P-450 isozymes, GSTs (α, μ and π) and EH activities can be further determined. AGE or SAC could be incubated with liver microsomes or fed to animals prior to the preparation of microsomes. Cytochrome P-450 activities could be determined as the metabolism of highly specific substrates as described in this study. Furthermore, microsomal proteins could be analysed by using Western blotting techniques with antibodies raised against P-450 isozymes, GST isoenzymes and EH. The mRNA levels of all three enzymes could also be determined using oligonucleotide or cDNA as probes by Northern blotting analyses.

(2) The GSH content of liver was increased by up to 80% of initial values during 7 days of pretreatment with AGE. The results presented in this study suggest that AGE and SAC decreased utilisation of GSH. The possible mechanism involving increased synthesis of GSH modulated by AGE, SAC and NAC should be further examined. The rates of GSH synthesis can be examined by adding AGE or SAC and a radiolabelled precursor of GSH (^{14}C-glycine or ^{14}C-cysteine) together into the incubation medium of liver slices. The activity of GSH synthetase could also be determined after feeding animals with AGE or SAC.

(3) It has been shown that SAC has a hepatoprotective effect *in vitro* against BB toxicity similar to that of AGE. The effect of SAC on BB-induced toxicity could be

further examined *in vivo* by feeding rats with SAC at doses equivalent to those used for AGE prior to the preparation of slices.

(4) The effects of feeding AGE or SAC to animals and then administering BB *in vivo* could also be investigated.

(5) AGE, SAC and NAC exhibited protective effects against BB-induced toxicity in the presence of buthionine sulphoximine (*BSO*). *It has* been suggested that these compounds are most likely to act by directly conjugating with BB-3,4-oxide. It may be possible to look for conjugates between SAC or other organosulphur compounds in AGE and the 3,4-oxide of BB by using ^{14}C-labelled bromobenzene. ^{14}C-BB would be added into the culture medium of liver slices in the presence or absence of AGE, SAC or NAC and then the profile of BB metabolites could be determined by HPLC analysis to look for differences in the profile of BB metabolites caused by AGE, SAC or NAC. The possible mechanism of reaction of SAC with BB-3,4-oxide could also be investigated using analogues of SAC.

(6) Paracetamol is a widely used analgesic and antipyretic that undergoes biotransformation reactions which are similar to those of BB. The P-450 derived electrophilic metabolites are trapped by GSH. According to 1993 data, over 94,000 exposures to paracetamol-containing medications in United States necessitated contact with poison control centres (Litovitz *et al.*, 1994). Thus, any protective effects of AGE and/or SAC against paracetamol toxicity towards liver slices may be of considerable importance and should be investigated in the future.

REFERENCES

Abdullah, T.H., Kandil, O., Elkadi, A. and Carter, J., 1988. Garlic revisited: therapeutic for the major diseases of our times? *Journal of The National Medical Association* **80**: 439-445.

Abuirmeileh, N., Yu, S.G., Qureshi, N., Lin, R.I.S. and Qureshi, A.A., 1991. Suppression of cholesterogenesis by Kyolic and S-allyl cysteine. *The FASEB Journal.* **5(6)**: A1756, #8048.

Acosta, D., Mitchell, D.B., Sorensen, E.M.B. and Bruckner, J.V., 1987. The metabolism and toxicity of xenobiotics in primary culture system of postnatal rat hepatocytes. In: The Isolated Hepatocyte: Use in Toxicity and Xenobiotic Biotransformations, (E.J.Rauckman and G.M.Padilla, eds.), Academic Press, Inc., pp. 189-213.

Agarwal, K.C., 1996. Therapeutic actions of garlic constituents. *Medicinal Research Reviews* **16**: 111-124.

Agius, L. and Tosh, D., 1990. Acinar zonation of cytosolic but not organelle-bound activities of phosphoenolpyruvate carboxykinase and aspartate aminotransferase in guinea-pig liver. *Biochemical Journal* **271**: 387-391.

Al-Mustafa, Z.H., Al-Ali, A.K., Qaw, F.S. and Abdul-Cader, Z., 1997. Cimetidine enhances the hepatoprotective action of N-acetylcysteine in mice treated with toxic doses of paracetamol. *Toxicology* **121**: 224-228.

Amagase, H. and Milner, J.A., 1993a. Impact of various sources of garlic and their constituents on 7,12-dimethylbenz[a]anthracene binding to mammary cell DNA. *Carcinogenesis* **14**: 1627-1631.

Amagase, H. and Milner, J.A., 1993b. Impact of dietary lipids on the ability of garlic to inhibit 7,12-dimethylbenz(a)anthracene (DMBA) binding to mammary DNA. *The FASEB Journal* **7**: A69.

Ames, B.N., 1971. The detection of chemical mutagens with enteric bacteria. *Chemical Mutagents: Princ. Methods Their Detect.* **1**: 267-283.

Ames, B.N., McCann, J. and Yamasaki, E., *1975. Methods for detecting carcinogens* and mutagens with the Salmonella/mammalian-microsome mutagenicity test. *Mutation Research* **31**: 347-364.

Anderson, M.E., 1985. Determination of glutathione and glutathione disulfide in biological samples. *Methods in Enzymology* **113**: 548-553.

Aprtz-Castro, R., Carrera, S., Cruz, M.R., Ledezma, E. and Jain, M.K., 1983. Effects of garlic extract and of three pure components isolated from it on human platelet

aggregation, arachidonate metabolism, release reaction and platelet ultrastructure. *Inhibition of Platelet Reaction* **32**: 155-169.

Azri, S., Gandolfi, A.J. and Brendel, K., 1990a. Precision-cut liver slices: an in vitro system for profiling potential hepatotoxicants. *In Vitro Toxicology. A Journal of Molecular and Cellular Toxicology* **3**: 309-320.

Azri, S., Gandolfi, A.J., Brendel, K., 1990b. Carbon tetrachloride toxicity in precision-cut rat liver slices. *In Vitro Toxicology. A Journal of Molecular and Cellular Toxicology* **3**: 127-138.

Azri, S., Mata, H.P., Gandolfi, A.J. and Brendel, K., 1992. The hepatotoxicity of chloroform in precision-cut rat liver slices. *Toxicology* **73(3)**: 239-250.

Barnabei, O., Leghissi, G. and Tomasi, V., 1974. Hormonal control of the potassium level in isolated rat liver cells. *Biochimia et Biophysica Acta* **362**: 316-325.

Barr, J., Weir, A.J., Brendel, K. and Sipes, I.G., 1991a. Liver slices in dynamic organ culture. I. An alternative in vitro technique for the study of rat hepatic drug metabolism. *Xenobiotica* **21**: 331-339.

Barr, J., Weir, A.J., Brendel, K. and Sipes, I.G., 1991b. Liver slices in dynamic organ culture. II. An in vitro cellular technique for the study of integrated drug metabolism using human tissue. *Xenobiotica* **21**: 341-350.

Baumann, A., Kerdar, R.S., Cramer, P., Feser, W., Blode, H., Salomon, A. and Kuhnz, W., 1996. Use of rat and human liver slices for the detection of steroid hormone-induced DNA-adducts in vitro by means of the ^{32}P-postlabeling technique. *Pharmacology & Toxicology* **78**: 214-223.

Baur, H., Kasperek, S. and Pfaff, E., 1975. Criteria of viability of isolated liver cells. *Hoppe-Seyler's Zeitschrift fuer Physiologische Chemie* **356**: 827-838.

Beamand, J.A., Price, R.J., Cunnighame, M.E. and Lake, B.G., 1993. Culture of precision-cut liver slices: effect of some peroxisome proliferators. *Food and Chemical Toxicology* **31**: 137-147.

Beutler, E., Duron, O. and Kelly, B.M., 1963. Improved method for the determination of blood glutathione. *Journal of Laboratory and Clinical Medicine* **1**: 882-888.

Blakely, S.R., Mislo, B.L., Brown, E.D., Jenkins, M.Y. and Mitchell, G.V., 1993. Gender differences in the induction of hepatic detoxification enzymes in garlic-fed hypercholesterolemic or iron-loaded mature rats. *The FASEB Journal* **7**: A864.

Boelsterli, U.A. and Goldlin, C., 1991. Biomechanisms of cocaine-induced hepatocyte injury mediated by the formation of reactive metabolites. *Archives of Toxicology* **65**: 351-361.

Boyer, J.L., Phillips, J.M. and Graf, J., 1990. Preparation and specific applications of *isolated hepatocyte couplets. Methods in Enzymology* **192**: 501-516.

Brady, J.F., Xiao, F., Wang, M.H., Li, Y., Ning, S.M., Gapac, *J.M. and Yang, C.S.*, 1991. Effects of disulfiram on hepatic P-450IIE1, other microsomal enzymes, and hepatotoxicity in rats. *Toxicology and Applied Pharmacology* **108**: 366-373.

Bridges, J.W., 1981. The use of hepatocytes in toxicological investigations. In: Toxicity Testing, (J.W.Gorrod, ed.), Taylor and Francis, Ltd., London. pp. 125-143.

Brightwell, R. and Tappel, A.L., 1968. Subcellular distributions and properties of rat liver phosphodiesterases. *Archives of Biochemistry and Biophysics* **124**: 325-332.

Brodeur, J. and Goyal, R., 1987. Effect of a cysteine prodrug, L-2-oxothiazolidine-4-carboxylic acid, on the metabolism and toxicity of bromobenzene: an acute study. *Canadian Journal of Physiology & Pharmacology* **65**: 816-822.

Brosche, T. and Platt, D., 1993. Zur immunomodulatorischen wirkung von knoblauch (*Allium sativum L.*). *Medizinische Welt* **44**: 309-313.

Brosche, T. and Platt, D., 1994. Knoblauchtherapie und zellulaere immurabwebr im alter. *Zeitschrift fuer Phytotherapie* **15**: 23-24.

Bruno, M.K., Cohen, S.D. and Khairallah, E.A., 1988. Antidotal effectiveness of N-acetylcysteine in reversing acetaminophen-induced hepatotoxicity. *Biochemical Pharmacology* **37**: 4319-4325.

Buben, J.A., Narasimnan, N. and Hanzlik, R.P., 1988. Effects of chemical and enzymic probes on microsomal covalent binding of bromobenzene and derivatives. Evidence for quinones as reactive metabolites. *Xenobiotica* **18**: 501-516.

Buege, J.A. and Aust, S.D., 1978. Microsomal lipid peroxidation. *Methods in Enzymology* **52**: 302-310.

Buening, M.K. and Franklin, M.R., 1976. SKF 525-A inhibition, induction, and 452-nm complex formation. *Drug Metabolism and Disposition* **4**: 244-255.

Burke, M.D., Thompson, S., Elcombe, C.R., Halpert, J., Haaparanta, T. and Mayer, R.T., 1985. Ethoxy-, pentoxy- and benzyloxyphenoxazones and homologues: A series of substrates to distinguish between different induced cytochromes P-450. *Biochemical Pharmacology* **34**: 3337-3345.

Campbell, A.K. and Hales, C.N., 1971. Maintenance of viable cells in an organ culture of mature rat liver. *Experimental Cell Research* **68**: 33-42.

Casini, A.F., Giorli, M., Hyland, R.J., Serroni, A., Gilfor, D. and Farber, J.L., 1982. Mechanisms of cell injury in the killing of cultured hepatocytes by bromobenzene. *Journal of Biological Chemistry* **257**: 6721-6728.

Casini, A.F., Pompella, A. and Comporti, M., 1985. Liver glutathione depletion induced by bromobenzene, idobenzene, and diethylmaleate poisoning and its relation to lipid peroxidation and necrosis. *American Journal of Pathology* **118**: 225-237.

Casini, A.F., Maellaro, E., Pompella, A., Ferrali, M. and Comporti, M., 1987. Lipid peroxidation, protein thiols and calcium homeostasis in bromobenzene-induced liver damage. *Biochemical Pharmacology* **36**: 3689-3695.

Casini, A.F., Maellaro, E., Del Bello, B. and Comporti, M., 1989. Lipid peroxidation and antioxidant systems in the liver injury induced by glutathione depleting agents. In: Free Radicals in the Pathogenesis of Liver Injury, (G.Poli, K.H.Cheeseman and M.U.Dianzani, eds.), Proceedings of the Second Congress on Free Radicals in Liver Injury, Oxford Pergamon. pp. 555-561.

Chung, I.S., 1985. Protective effect of garlic on ethanol-induced gastric mucosal injury in rats. *Journal. Catholic Medical College* **38**: 1211-1224.

Connors, S., Rankin, D.R., Gandolfi, A.J., Krumdieck, C.L., Koep, L.J. and Brendel, K., 1990. Cocaine hepatotoxicity in cultured liver slices: a species comparison. *Toxicology* **61**: 171-183.

Corcoran, G.B. and Wong, B.K., 1986. Role of glutathione in prevention of acetaminophen-induced hepatotoxicity by N-acetyl-L-cysteine in vivo: studies with N-acetyl-D-cysteine in mice. *Journal of Pharmacology and Experimental Therapeutics* **238**: 54-61.

Crook, T.R., Souhami, R.L., Whyman, G.D. and McLean, A.E.M., 1986. Glutathione depletion as a determinant of sensitivity of human leukemia cells to cyclophosphamide. *Cancer Research* **46**: 5035-5038.

Dagani, R., 1984. In vitro methods may offer alternatives to animal testing. *Chemical & Engineering News* **12**: 25-28.

Dai, Y. and Cederbaum, A.I., 1995. Cytotoxicity of Acetaminophen in human cytochrome P4502E1-transfected HepG2 Cells. *The Journal of Pharmacology and Experimental Therapeutics* **273**: 1497-1505.

Dale, D., Gandolfi, A.J., Brendel, K. and Schuman, S., 1988. Rat liver slices and diazepam metabolism: in vitro interactions with volatile anaesthetic drugs and albumin. *British Journal of Anaesthesia* **60**: 692-696.

Dalvi, R.R., 1992. Alterations in hepatic phase I and phase II biotransformation enzymes by garlic oil in rats. *Toxicology Letters* **60**: 299-305.

Dankovic, D.A. and Billings, R.E., 1984. Bromobenzene toxicity and metabolism in isolated hepatocytes. *Toxicologist* **4**: Abstract.

Dankovic, D.A. Billings, R.E., Seifert, W. and Stillwell, W.G., 1985. Bromobenzene metabolism in isolated rat hepatocytes. $^{18}O_2$ incorporation studies. *Molecular Pharmacology* **27**: 287-295.

Dankovic, D.A. and Billings, R.E., 1985. The role of 4-bromophenol in bromobenzene covalent binding and toxicity in isolated rat hepatocytes. *Toxicology and Applied Pharmacology* **79**: 323-331.

Dausch, J.G. and Nixon, D.W., 1990. Garlic: a review of its relationship to malignant disease. *Preventive Medicine* **19**: 346-361.

Davis, D.C., Hishimoto, M. and Gillette, J.R., 1973. Effects of bromobenzene and carbon tetrachloride on the synthesis and release of proteins by perfused rat liver. *Biochemical Pharmacology* **22**: 1989-2001.

Davis, L.F., Shen, J.K. and Royer, R.E., 1994. In vitro synergism of concentrated *Allium sativum* extract and amphotericin B against cryptococcus neoformans. *Planta Medica* **60**: 546-549.

Day, B.J., DeNicola, D.B. and Carlson, G.P., 1992. Potentiation of bromobenzene-induced pheumotoxicity by phenobarbital as determined by bronchoalveolar lavage fluid analysis. *Drug and Chemical Toxicology* **15(1)**: 33-51.

Deas, O., Dumont, C., Mollereau, B., Metivier, D., Pasquier, C., Bernard-Pomier, G., Hirsch, F., Charpentier, B. and Senik, A., 1997. Thiol-mediated inhibition of FAS and CD2 apoptotic signalling in activated human peripheral T cells. *International Immunology* **9**: 117-125.

Dickson, A.J. and Pogson, C.I., 1977. The metabolic integrity of hepatocytes in sustained incubations. *FEBS Letters* **83**: 27-32.

Dillard, C.S. and Tappel, A.L., 1989. Lipid peroxidation products in biological tissues. *Free Radical Biology and Medicine* **7(2)**: 193-196.

Dion, M.E. and Milner, J.A., 1996. Formation and bioactivation of nitrosomorpholine is inhibited by S-allyl cysteine. *The FASEB Journal* **10**: 2869.

Dogterom, P., 1993. Development of a simple incubation system for metabolism studies with precision-cut liver slices. *Drug Metabolism and Disposition* **21**: 699-704.

Dorant, E., Vanden Brandt, P.A. and Goldbohm, R.A., 1993. Garlic and its significance for the prevention of cancer in humans: a critical view. *British Journal of Cancer* **67**: 424-429.

Dragnev, K.H., Nims, R.W. and Lubet, R.A., 1995. The chemmopreventive agent diallyl sulfide. A structurally atypical phenobarbital-type inducer. *Biochemical Pharmacology* **50**: 2099-2104.

Drew, R. and Miners, J.O., 1984. The effects of buthionine sulphoximine (BSO) on glutathione depletion and xenobiotic biotransformation. *Biochemical Pharmacology* **33**: 2989-2994.

Droge, W., 1993. Cysteine and glutathione deficiency in AIDS patients: a rationale for the treatment with N-acetylcysteine. *Pharmacology* **46**: 61-65.

Dunn, M.J. and Bradd, S.J., 1993. Separation and analysis of membrane proteins by SDS-polyacrylamide gel electrophoresis. *In:* Methods in Molecular Biology, (J.M.Graham and J.A.Higgins, eds.), Human Press Inc. *19: 203-210.*

Duthie, S.J., Melvin, W.J and Burke, M.D., 1994. Bromobenzene detoxification in the human liver-derived HepG2 cell line. *Xenobiotica* **24**: 265-279.

Edwards, A.M., Glistak, M.L., Lucas, C.M. and Wilson, P.A., 1984. 7-Ethoxycoumarin-O-deethylase activity as a convenient measure of liver drug metabolizing enzymes: regulation in cultured rat hepatocytes. *Biochemical Pharmacology* **33**: 1537-1546.

Fakjian, N. and Buckpitt, A.R., 1984. Metabolism of bromobenzene to glutathione adducts in lung slices for mice treated with pheumotoxicants. *Biochemical Pharmacology* **33**: 1479-1486.

Federoff, S., 1967. Proposed usage of animal tissue culture terms. *Journal of the National Cancer Institute* **38**: 607-611.

Ferrandiz, M.L., Bustos, G., Paya, M., Gunasegaran, R. and Alcaraz, M.J., 1994. Hispidulin protection against hepatotoxicity induced by bromobenzene in mice. *Life Sciences* **55(8)**: PL145-PL150.

Fisher, R.L., Smith, P.F., Sipes, I.G., Gandolfi, A.J., Krumdieck, C.L. and Brendel, K., 1990a. Toxicity of chlorobenzenes in cultured rat liver slices. *In Vitro Toxicology. A Journal of Molecular and Cellular Toxicology* **3**: 181-194.

Fisher, R.L., McCarthy, S., Sipe, I.G., Hanzlik, R.R. and Brendel, K., 1990b. Metabolism of dichlorobenzenes in organ cultured liver slices. *Advances in Experimental Medicine and Biology* **283**: 717-723.

Fisher, R.L., Nau, H., Gandolfi, A.J. and Brendel, K., 1991a. Toxicity of valproic acid in liver slices from Sprague-Dawley rats and domestic pigs. *Toxicology In Vitro* **5**: 201-205.

Fisher, R.L., Barr, J., Zukoski, C.F., Putnam, C.W., Sipes, I.G., Gandolfi, A.J. and Brendel, K., 1991b. In vitro hepatotoxicity of three dichlorobenzene isomers in human liver slices. *Human & Experimental Toxicology* **10**: 357-363.

Fisher, R.L., Hanzlik, R.P., Gandolfi, A.J. and Brendel, K., 1991c. Toxicity of ortho-substituted bromobenzenes in rat liver slices: a comparison to isolated hepatocytes and the whole animal. *In Vitro Toxicology* **4**: 173-186.

Fisher, R.L., Brendel, K. and Hanzlik, R.P., 1993. Correlation of metabolism, covalent binding and toxicity for a series of bromobenzene derivations using rat liver slices in vitro. *Chemco-Biological Interactions* **88(2-3)**: 191-198.

Fisher, R.L., Sanuik, J.T., Nau, H., Gandolfi, A.J. and Brendel, K., 1994. Comparative toxicity of valproic acid and its metabolites in liver slices from adult rats, weanling rats and humans. *Toxicology in Vitro* **8**: 371-379.

Fisher, R.L., Hasal, S.J., Sanuik, J.T., Gandolfi, A.J. and Brendel, K., 1995a. Determination of optimal incubation media and suitable slice diameters in precision-cut liver slices: optimization of tissue slice culture, part 2. *Toxicology Methods* **5**: 115-130.

Fisher, R.L., Shaughnessy, R.R., Jenkins, P.M., Austin, M.L., Roth, G.L., Gandolfi, A.J. and Brendel, K., 1995b. Dynamic organ culture is superior to multiwell plate culture for maintaining precision-cut tissue slice: optimization of tissue slice culture, part 1. *Toxicology Methods* **5**: 99-113.

Flohe, L., Guenzler, W.A. and Ladenstein, R., 1976. Glutathione peroxidase. In: Glutathione: Metabolism and Function, (I.M.Arias and W.B.Jacoby, eds.), Raven Press/New York, pp. 115-138.

Freshney, R.I., 1983. Culture of *Animals Cells* - a Manual of Basic Technique, (R.I.Freshney, ed.), Alan R. Liss, Inc., New York, pp. *225-235.*

Gad, S.C., 1994. In Vitro Toxicology, (S.C.Gad, ed.), Raven Press/New York.

Gebhardt, R., 1995. Application of palmitate-induced inhibition of cholesterol biosynthesis in cultured rat hepatocytes by garlic-derived organosulfur compounds. *Phytomedicine* **2(1)**: 29-34.

Geng, Z. and Lau, B.H.S., 1997. Aged garlic extract modulates glutathione redox cycle and superoxide dismutase activity in vascular endothelial cells. *Phytotherapy Research* **11**: 54-56.

Ghantous, H.N., Fernando, J., Gandolfi, A.J. and Brendel, K., 1990a. Minimal biotransformation and toxicity of desflurance in guinea-pig liver slices. *Drug Metabolism Disposition* **18**: 514-518.

Ghantous, H.N., Fernando, J., Morgan, S.E., Gandolfi, A.J. and Brendel, K., 1990b. Precision-cut guinea pig liver slices as a tool for studying the toxicity of volatile anaesthetics. *ATLA*. **18**: 191-199.

Ghanotous, H.N., Fernando, J., Gandolfi, A.J. and Brendel, K., 1991a. Biotransformation of halothane in guinea-pig liver slices. *Anesthesia and Analgesia* **72**: 796-800.

Ghantous, H.N., Fernando, J., Gandolfi, A.J. and Brendel, K., 1991b. Inhibition of protein synthesis and secretion by volatile anaesthetics in guinea pig liver slices. *Advances in Experimental Medicine and Biology* **283**: 725-730.

Gibson, G.G. and Skett, P., 1994. Introduction to Drug Metabolism, Blackie Academic & Professional, 2rd edition.

Giri, S. and Siegel, D.M., 1990. Role of mixed-function oxidases and non-protein sulphydryl content in [^{14}C]-2-chloro-4-acetotoluidide binding to liver and kidney in starlings. *Journal of Applied Toxicology* **10(6)**: 429-438.

Glodkner, R. and Muller, D., 1995. Ethoxycoumarin O-deethylation (ECOD) activity in rat liver slices exposed to beta-naphthoflavone (BNF) in vitro. *Experimental Toxicologic Pathology* **47**: 319-324.

Goldstein, A., Aronow, L. and Kalman, *S.M., 1973. Principles of Drug* Action. The Basis of Pharmacology, (J.Wiley and Sons, eds.), 2nd edition, New York.

Gonzalez, F.J., 1990. Molecular genetics of the P450 superfamily. *Pharmacology Therapeutics* **45**: 1-38.

Gonzalez, F.J., 1992. Human cytochromes P-450: problems and perspectives. *Trends Pharmacological Sciences* **13**: 346-352.

Gottschall, D.W., Harder, R.R., Wiley, R.A. and Hanzlik, P.P., 1984. Chemical models for toxic metabolites of bromobenzene derivatives. Relative toxicity toward isolated hepatocytes. *Toxicology* **31**: 251-259.

Greenlee, W.F. and Poland, A., 1978. An improved assay of 7-ethoxycoumarin-O-deethylase activity: induction of hepatic enzyme activity in C57BL/6J and DBA/2J mice by phenobarbital, 3-methylcholanthrene and 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Journal of Pharmacology Experimental Therapeutics* **205**: 596-605.

Grewal, K.K., Rafeiro, E. and Racz, W.J., 1996. Bromobenzene and furosemide hepatotoxicity: alterations in glutathione, protein thiols, and calcium. *Canadian Journal of Physiology and Pharmacology* **74**: 257-264.

Griffith, O.W., 1982. Mechanism of action, metabolism, and toxicity of buthionine sulfoximine and its higher homologs, potent inhibitors of glutathione synthesis. *The Journal of Biological Chemistry* **257**: 13704-13712.

Grisham, J.W., Charlton, R.K. and Kaufman, D.G., 1978. In vitro assay of cytotoxicity with cultured liver: accomplishments and possibilities. *Environmental Health Perspectives* **25**: 161-171.

Grisham, J.W., 1979. Use of hepatic cell cultures to detect and evaluate the mechanisms of action of toxic chemicals. *International Review of Experimental Pathology* **20**: 123-209.

Grune, T., Scherat, T., Behrend, H., Conradi, E., Brenke, R. and Siems, W., 1996. Influences of allium sativum on oxidative stress status - a clinical investigation. *Phytomedicine* **2(3)**: 205-207.

Gudi, V.A. and Singh, S.V., 1991. Effect of diallyl sulfide, a naturally occurring anti-carcinogen on glutathione-dependent detoxification enzymes of female CD-mouse tissue. *Biochemical Pharmacology* **42**: 1261-1265.

Guillouzo, A. and Guguen-Guillouzo, C., 1992. In vitro approaches to hepatotoxicity studies. In: In Vitro Methods in Toxicology, (G.Jolles and A.Cordier, eds.), Academic Press Ltd., pp. 131-163.

Guyonnet, D., Siess, M.H., Le Bon, A.M. and Suschetet, M., 1999. Modulation of phase II enzymes by organosulfur compounds from allium vegetables in rat tissues. *Toxicology and Applied Pharmacology* **154**: 50-58.

Gwilt, P.R., Lear, C.L., Tempero, M.A, Birt, D.D., Grandjean, A.C., Ruddon,R.W. and Nagel, D.L., 1994. The effect of garlic extract on human metabolism of acetaminophen. *Cancer Epidemiology, Biomarkers & Prevention* **3**: 155-160.

Gwynn, J., Fry, J.R. and Bridges, J.W., 1979. The effect of paracetamol and other foreign compounds in protein synthesis *in isolated adult rat hepatocytes*. *Biochemical Society Transactions* **7**: 117-118.

Haber, D., Siess, M.H., De Waziers, I., Beaune, P. and Suschetet, M., 1994. Modification of hepatic drug-metabolizing enzymes in rat fed naturally occurring allyl sulphides. *Xenobiotica* **24**: 169-182.

Hammond, A.H. and Fry, J.R., 1996. Effects of culture duration, cytochrome P-450 inhibition and glutathione depletion on toxicity of diverse xenobiotics. *Toxicology in Vitro* **10**: 315-321.

Hansen, S.H., Bjornsdottir, I., Bager, S. and Poulsen, M.N., 1993. Nyt stoff til hvidlogsdebatten. *Farmaci* **100**: 6-8.

Harauchi, T. and Hirata, M., 1994. Effect of P-450 inducers of glutathione (GSH) depletion by bromobenzene in primary cultures of dog hepatocytes. *Biological & Pharmaceutical Bulletin* **17(5)**: 658-661.

Harman, A.W. and Self, G., 1986. Comparison of the protective effects of N-acetylcysteine, 2-mercaptopropionylglycine and dithiothreitol against acetaminophen toxicity in mouse hepatocytes. *Toxicology* **41**: 83-93.

Hart, A., Mattheyse, F.J. and Balinsky, J.B., 1983. An organ culture of postnatal rat liver slices. *In Vitro* **19**: 841-852.

Hatono, S., Jiminez, A. and Wargovich, M.J., 1996. Chemopreventative effect of S-allyl cysteine and its relationship to the detoxification enzyme glutathione S-transferase. *Carcinogenesis* **17**: 1041-1044.

Hayes, M.A., Roberts, E., Roomi, M.W., Safe, S.H., Farber, E. and Cameron, R.G., 1984. Comparative influences of different PB-type and 3-MC-type polychlorinated

biphenyl-induced phenotypes or cytocidal hepatotoxicity of bromobenzene and acetaminophen. *Toxicology Applied Pharmacology* **76**: 118-127.

Hayes, M.A. and Pickering, D.B., 1985. Comparative cytopathology of primary rat hepatocyte cultures exposed to aflatoxin B_1, acetaminophen, and other hepatotoxins. *Toxicology and Applied Pharmacology* **80**: 345-356.

Hayes, M.A., Murray, C.A. and Rushmore, T.H., 1986. Influences of glutathione status on different cytocidal responses of monolayer rat hepatocytes exposed to aflatoxin B_1 or acetaminophen. *Toxicology Applied Pharmacology* **85**: 1-10.

Hikino, H., Tohkin, M., Kiso, Y., Namiki, T., Nishimura, S. and Takeyama, K., 1986. Antihepatotoxic actions of allium sativum bulbs. *Planta Medica* **52**: 163-168.

Hinson, J.A. and Roberts, D.W., 1992. Role of covalent and noncovalent interactions in cell toxicity: effects on protein. *Annual Review of Pharmacology and Toxicology* **32**: 471-510.

Hinton, R.H. and Grasso, P., 1993. Hepatotoxicity. In: General and Applied Toxicity, Vol. 1, (B.Ballantyne, T.Marr and P.Turner, eds.), Stockton Press. pp. 619-662.

Hirao, Y., Sumioka, I., Nakagami, S., Yamamoto, M., Hatono, S., Yoshida, S., Fuwa, T. and Nakagawa, S., 1987. Activation of immunoresponder cells by the protein fraction from aged garlic extract. *Phytotherapy Research* **1**: 161-164.

Hodges, G.M., 1975. Review of methodology in organ culture. In: Organ Culture in Biomedical Research, (*M.Balls and M.A.Monnichendam*, eds.), Cambridge University Press, New York,

Holm-Hansen, O. and Karl, D.M., 1978. Biomass and adenylate energy charge determination in microbial cell extracts and environmental samples. *Methods in Enzymology* **17**: 73-85.

Horie, T., Murayama, T., Mishima, T., Ito, F., Minamide, Y., Fuwa, T. and Awazu, S., 1989. Protection of liver microsomal membranes from lipid peroxidation by garlic extract. *Planta Medica* **55**: 506-508.

Horie, T., Awazu, S., Itakura, Y. and Fuwa, T., 1992. Identified diallyl polysulfides from an aged garlic extract which protects the membranes from lipid peroxidation. *Planta Medica* **58**: 468-469.

Hu, J.J., Yoo, J.S., Lin, M., Wang, E.J. and Yang, C.S., 1996a. Protective effects of diallylsulphide on acetaminophen-induced toxicities. *Food Chemical Toxicology* **34**: 963-969.

Hu, X., Benson, P.J., Srivastava, S.K., Mack, L.M., Xia, H., Gupta, V., Zaren, H.A. and Singh, S.V., 1996b. Glutathione S-transferases of female A/J mouse liver and forestomach and their differential induction by anti-carcinogenic organosulfides from garlic. *Archives of Biochemistry Biophysics* **336**: 199-214.

Hu, X. and Singh, S.V., 1997. Glutathione S-transferases of female A/J mouse lung and their induction by anticarcinogenic organosulfides from garlic. *Archives of Biochemistry and Biophysics* **340**: 279-286.

Hue, D.P., Griffith, K.L. and McLean, A.E.M., 1985. Hepatocytes in primary culture become susceptible to paracetamol injury after depletion of glutathione using DL-buthionine-SR-sulphoximine (BSO). *Biochemical Pharmacology* **34**: 4341-4344.

Ichihara, A., Nakamura, T. and Tannka, K., 1982. Use of hepatocytes in primary culture for biochemical studies on liver functions. *Molecular and Cell Biochemistry* **43**: 145-160.

Ide, N., Matsuura, H. and Itakura, Y., 1996. Scavenging effects of aged garlic extract and its constituents on active oxygen species. *Phytotherapy Research* **10**: 340-341.

Imai, J., Ide, N., Nagae, S., Moriguchi, T., Matsuura, H. and Itakura, Y., 1994. Antioxidant and radical scavenging effects of aged garlic extract and its constituents. *Planta Medica* **60**: 417-420.

Ishikawa, K., Naganawa, R., Yoshida, H., Iwatu, N., Fukuda, H., Fujino, T. and Suzuki, A., 1996. Antimutagenic effects of ajoene, an organosulfur compound derived from garlic. *Biosciences, Biotechnology and Biochemistry* **60(12)**: 2086-2088.

Jewell, S.A., Bellomo, G., Thor, H. and Orrenius, S., 1982. Bleb formation in hepatocytes during drug metabolism is caused by disturbances in thiol and calcium ion homeostasis. *Science (Washington D.C)* **217**: 1257-1259.

Jin, L. and Baillie, T.A., 1997. Metabolism of the chemoprotective agent diallyl sulfide to glutathione conjugates in rats. *Chemical Research Toxicology* **10**: 318-327.

Jollow, D.J., Mitchell, J.R., Zampaglione, N. and Gillette, J.R., 1974. Bromobenzene-induced liver necrosis. Protective role of glutathione and evidence for 3,4-bromobenzene oxide as the hepatotoxic metabolite. *Pharmacology* **11**: 151-169.

Jordan, R.A. and Schenkman, J.B., 1982. Relationship between malondialdehyde production and arachidonate consumption during NADPH-supported microsomal lipid peroxidation. *Biochemical Pharmacology* **31(7)**: 1393-1400.

Jungermann, K. and Katz, N., 1989. Functional specialisation of different hepatocyte populations. *Physiological Reviews* **69**: 208-264.

Jurima-Romet, M., Huang, H.S., Paul, C.J. and Thomas, B.H., 1991. Enalapril cytotoxicity in primary cultures of rat hepatocytes. II. Role of glutathione. *Toxicology Letters* **58**: 269-277.

Jurima-Romet, M., Abbott, F.S., Tang, W., Huang, H.S. and Whitehouse, L.W., 1996. Cytotoxicity of unsaturated metabolites of valproic acid and protection by vitamins C and E in glutathione-depleted rat hepatocytes. *Toxicology* **112**: 69-85.

Kagawa, K., Masutaka, H. and Yamaguchi, Y., 1986. Garlic extract inhibits the enhanced peroxidation and production of lipids in carbon tetrachloride-induced liver injury. *Japanese Journal of Pharmacology* **42**: 19-26.

Kiesewetter, H. and Jung, F., 1992. Kann knoblauch die atherosklerose verhindern? Argynebte and Fakten, pp. 6-10.

Kim, S.G., Ham, S.Y., Chung, H.C, Hong, S.Y. and Jung, K.H., 1995. Enhanced effectiveness of dimethyl-4,4'-demethoxy-5,6,5',6'-demethylene dioxybiphenyl-2,2'-dicarboxylate in combination with garlic oil against experimental hepatic injury in rats and mice. *Journal Pharmacy Pharmacology* **47**: 678-682.

Kim, S.G., Chung, H.C and Cho, J.Y., 1996. Molecular mechanism for alkyl sulfide-modulated carbon tetrachloride-induced hepatotoxicity: the role of cytochrome P4502E1, P4502B and glutathione S-transferase expression. *The Journal of Pharmacology and Experimental Therapeutics* **277**: 1058-1066.

Klaassen, C.D. and Stacey, N.H., 1982. Use of isolated hepatocytes in toxicity assessment. In: Toxicology of the Liver, (G.Plaa and W.Hewitt, eds.), Raven Press, New York. pp. 147-179.

Kleijnen, J., Knipschild, P. and Terriet, G., 1989. Garlic, onions and cardiovascular risk factor. A review of the evidence from human experiments with emphasis on commercially available preparation. *British Journal Clinical Pharmacology* **28**: 535-544.

Klingele, H.O., 1973. Biotransformation. In: Absorption, Distribution, Transformation and Excretion of Drugs, (P.K.Knoeffel, ed.), Thomas, Springfield, pp. 77-108.

Koch, H.P., 1989. Kann man knoblauch "geruchlos" machen? *Deutsche Apotheker-Zeitung* **129**: 1991-1996.

Koch, H.P. and Lawson, L.D., 1996. Garlic. The Science and Therapeutic Application of Allium sativum L. and Related Species, (H.P.Koch and L.D.Lawson, eds.), second edition, Williams & Wilkins.

Kojima, R., Toyama, Y. and Ohnishi, S.T., 1994. Protective effects of an aged garlic extract on doxorubin-induced cardiotoxicity in the mouse. *Nutrition and Cancer* **22**: 163-173.

Kosower, N.S. and Kosower, E.M., 1978. The glutathione status of cells. *International Review of Cytology* **54**: 109-160.

Krack, G., Goethals, F., Deboyser, D. and Roberfroid, M., 1983. Metabolic competence of isolated hepatocytes in suspension: A new tool for in vitro toxicological evaluation. In: Isolation, Characterization, and Use of Hepatocytes, (R.A.Harris and N.W.Cornell, eds.), Elsevier, New York, pp. 391-398.

Krack, G., Deboyser, D., Goethals, F., Vossen, P. and Roberfroid, M., 1985. An in vitro model for acute toxicity testing using hepatocytes freshly isolated from adult mammals.

In: Safety Evaluation and Regulation of Chemicals. II. Impact of Regulations: Improvements of Methods, (F.Homburger, ed.), pp. 286-294.

Krebs, H.A., Cornell, N.W., Lund, P. and Hems, R., 1974. Isolated liver cells as experimental material. In: Regulation of Hepatic Metabolism, (F.Lundquist and N.Tygstrup, eds.), Munksgaard, Copenhagen, pp. 726-750.

Krebs, H.A., Lund, P. and Edwards, M., 1979. Criteria of metabolic competence of isolated hepatocytes. In: Cell Populations, (E.Reed, ed.), Ellis Howard, Chichester, p. 1.

Kwak, M.K., Kim, S.G. and Kim, N.D., 1995. Effects of garlic oil on rat hepatic P4502E1 expression. *Xenobiotica* **25**: 1021-1029.

Lake, B.G., 1987. Preparation and characterisation of microsomal fractions for studies on xenobiotic metabolism. In: Biochemical Toxicology: a practical approach, (K.Snell and B.Mullock, eds.), IRL Press, pp. 183-215.

Lake, B.G., Beamend, J.A., Japenga, A.C., Renwick, A., Davies, S. and Price, R.J., 1993. Induction of cytochrome-P-450-dependent enzyme-activities in cultured rat liver slices. *Food and Chemical Toxicology* **31**: 377-386.

Lau, S.S, Monks, T.J. and Gillette, J.R., 1984. Multiple reactive metabolites derived from bromobenzene. *Drug Metabolism and Disposition* **12**: 291-296.

Lau, S.S. and Monks, T.J., 1988. Minireview: The contribution of bromobenzene to our current understanding of chemically-induced toxicities. *Life Sciences* **42**: 1259-1269.

Lauterburg, B.H., Corcoran, G.B. and Mitchell, J.R., 1983. Mechanism of action of N-acetylcysteine in the protection against the hepatotoxicity of acetaminophen in rats in vivo. *Journal of Clinical Investigation* **71**: 980-991.

Lawson, L.D., Ranson, D.K. and Hughes, B.G., 1992. Inhibition of whole blood platelet-aggregation by compounds in garlic clove extracts and commercial garlic products. *Thrombosis Research* **65**: 141-156.

Lawson, L.D., 1993. Bioactive organosulfur compounds of garlic and garlic products: role in reducing blood lipids. In: Human Medicinal agents from plants, (A.D.Kinghorn and M.F.Balandrin, eds.), Am. Chem. Soc. Books, Washington, D.C., pp. 306-330.

Lawson, L.D. and Wang, Z.J., 1993. Pre-hepatic fate of the organosulfur compounds derived from garlic (*Allium sativum*). *Planta Medica* **59**: A688.

Lawson, L.D., 1996. Garlic: A review of its biological effects and indicated active compounds. *Abstracts of Papers of the* American *Chemical Society* **212**, PT1, pp. 91.

Le Douarin, G., 1970. Differentiation of organs in natural media. In: Organ Culture, (J.A.Thomas, ed.), New York and London, Academic Press, pp. 13-70.

Leevy, C.M, Quinlan, D.P. and Leevy, C.B., 1980. Evaluation of toxic liver injury. In: Toxic Injury of the Liver-Part B, (E.Farber and M.M.Fisher, eds.), Marcel Dekker, Inc., New York, p. 488.

Legator, M.S. and Malling, H.V., 1971. The host-mediated assay, a practical procedure for evaluating potential mutagenic agents in mammals. *Chemical Mutagens: Princ. Methods Their Detect.* **2**: 569-589.

Li, A.P., 1994. Primary hepatocyte culture as an in vitro toxicological system of the liver. In: In Vitro Toxicology, (S.C.Gad, ed.), Raven Press/New York, pp. 195-220.

Li, G., Qiao, C.H., Lin, R.I., Pinto, J.T., Osborne, M.P. and Tiwari, D.K., 1995. Anti-proliferative effects of garlic constituents in cultured human breast cancer cell. *Oncology Reports* **2**: 787-791.

Liberti, J.P., Du Valls, C.H. and Wood, D.M., 1971. In vitro induction of tyrosine aminotransferase in liver slices by hydrocortisone. *Canadian Journal of Biochemistry* **49**: 1357-1361.

Lin, M.C., Wang, E.J., Patten, C., Lec, M.J., Xiao, F., Reuhl, K.R. and Yang, C.S., 1996. Protective effect of diallyl sulfone against acetaminophen-induced hepatotoxicity in mice. *Journal of Biochemical Toxicology* **11**: 11-20.

Litovitz, T.L., Clark, L.R. and Soloway, R.A., 1994. 1993 annual report of the American Association of poison control centres toxic exposure surveillance system. *American Journal of Emergency Medicine* **12**: 546-584.

Liu, J., Liu, Y.P., Bullock, P. and Klaassen, C.D., 1995. Suppression of liver cytochrome P450 by 2-hederin: relevance to hepatoprotection. *Toxicology and Applied Pharmacology* **134**: 124-131.

Locke, S.J. and Brauer, M., 1991. The response of the rat liver *in situ* to bromobenzene-*in vivo* proton magnetic resonance imaging and P31 magnetic resonance spectroscopy studies. *Toxicology and Applied Pharmacology* **110**: 416-428.

Lu, F.C., 1985. Basic Toxicology: Fundamentals, Target Organs, and Risk Assessment. Hemisphere Publishing Corporation, pp. 21-39.

Lubet, R.A., Nims, R.W., Mayer, R.T., Cameron, J.W. and Schechtman, L.M., 1985. Measurement of cytochrome P-450 dependent dealkylation of alkoxy-phenoxazones in hepatic S9s and hepatocyte homogenates: effects of dicumarol. *Mutation Research* **142**: 127-131.

Maellaro, E., Del Bello, B., Casini, A.F, Comporti, M., Ceccarelli, D., Muscatello, U. and Masini, A., 1990. Early mitochondrial dysfunction in bromobenzene treated mice: a possible factor of liver injury. *Biochemical Pharmacology* **40(7)**: 1491-1497.

Margulis, L., 1973. Colchicine-sensitive microtubules. *International Review of Cytology* **34**: 333-361.

Martin, F.L. and McLean, A.E.M., 1995. Adenosine triphosphate (ATP) levels in paracetamol-induced cell injury in the rat in vivo and in vitro. *Toxicology* **104**: 91-97.

Maslansky, C.J. and Williams, G.M., 1982. Primary cultures and the levels of cytochrome P450 in hepatocytes from mouse, rat, hamster and rabbit liver. *In Vitro* **18**: 683-693.

Massey, T.E. and Racz, W.J., 1981. Effects of N-acetylcysteine on metabolism, covalent binding, and toxicity of acetaminophen in isolated mouse hepatocytes. *Toxicology and Applied Pharmacology* **60**: 220-228.

McLellan, L., Lewis, A.D., Hall, D.J., Ansell, J.D. and Wolf, C.R., 1995. Uptake and distribution of N-acetylcysteine in mice: tissue-specific effects on glutathione concentrations. *Carcinogenesis* **16**: 2099-2106.

McLimans, W.F., 1972. The gaseous environment of the mammalian cell in culture. In: Growth, Nutrition and Metabolism of Cells in Culture, (G.H.Rothblatt and V.J.Gristofalo, eds.), New York and London, Academic Press, pp. 137-170.

McMahon, F.G. and Vargas, R., 1993. Can garlic lower blood pressure? A pilot study. *Pharmacolotheraphy* **13**: 406-407.

McQeen, C.A., 1988. Hepatocytes in monolayer culture: an in vitro model for toxicity study. In: In Vitro Toxicology: Model Systems and Methods, (C.A.McQeen, ed.), Telford Press, Inc., New York.

Medzihrasky, F. and Marks, M.J., 1975. Measures of viability of isolated cells. *Biochemical Medicine* **13**: 164-177.

Meister, A. and Anderson, M.E., 1983. Glutathione. *Annual Review of Biochemistry* **52**: 711-760.

Mennes, W.C., van Holsteijn, C.W.N., Noordhoek, J. and Blaauboer, B.J., 1991. Comparative cytotoxicity of bromobenzene in primary cultures of rat and hamster hepatocytes and its relation to biotransformation. *Toxicology in Vitro* **5(1)**: 63-70.

Mennes, W.C., van Holsteijn, C.W.N., van Iersei, A.A.J., Yap, S.H., Noordhoek, J. and Blaauboer, B.J., 1994. Interindividual variation in biotransformation and cytotoxicity of bromobenzene as determined in primary hepatocyte cultures derived from monkey and human liver. *Human & Experimental Toxicology* **13**: 415-421.

Meredith, M.J. and Reed, D.J., 1982. Status of the mitochondrial pool of glutathione in the isolated hepatocyte. *Journal of Biological Chemistry* **257**: 3747-3753.

Mertens, K., Kaufman, S., Waterschoot, S., Vercruysse, A. and Rogiers, V., 1996. Effect of teriary Butylhydroperoxide-induced oxidative stress on glutathione content and thiobarbituric acid reactive substances production in cultures and co-cultures of adult rat hepatocytes. *Toxicology in Vitro* **10**: 507-511.

Miller, M.G., Beyer, J., Hall, G.L., De Graffenried, L.A. and Adams, P.E., 1993. Predictive value of liver slices for metabolism and toxicity in vivo use of acetaminophen as a model hepatotoxicant. *Toxicology and Applied Pharmacology* **122**: 108-116.

Miners, J.O., Drew, R. and Birkett, D.J., 1984. Mechanism of action of paracetamol protective agents in mice in vivo. *Biochemical Pharmacology* **33**: 2995-3000.

Mizutani, T., Nakahori, Y. and Yamamoto, K., 1994. p-Dichlorobenzene-induced hepatotoxicity in mice depleted of glutathione by treatment with buthionine sulfoximine. *Toxicology* **94**: 57-67.

Monks, T.J. and Lau, S.S., 1984. Activation and detoxification of bromobenzene in extrahepatic tissues. *Life Sciences* **35**: 561-568.

Moriguchi, T., Takashina, K. and Chu, P., 1994. Prolongation of life span and improved learning in the senescence accelerated mouse produced by aged garlic extract. *Biological & Pharmaceutical Bulletin* **117**: 1589-1594.

Moriguchi, T., Saito, H. and Nishiyama, N., 1996a. Aged garlic extract prolongs longevity and improves spatial memory deficit in senescence-accelerated mouse. *Biological & Pharmaceutical Bulletin* **19(2)**: 305-307.

Moriguchi, T., Nishiyama, N., Saito, H. and Katsuki, H., 1996b. Trophic effects of aged garlic extract (AGE) and its fractions on primary cultured hippocampal neurons from fetal rat brain. *Phytotherapy Research* **10**: 468-472.

Moriguchi, T., Saito, H. and Nishiyama, N., 1997. Anti-ageing effect of aged garlic extract in the inbred brain atrophy mouse model. *Clinical and Experimental Pharmacology and Physiology* **24**: 235-242.

Morris, J., Burke, V., Mori, T.A., Vandongen, R. and Beilin, L., 1995. Effects of garlic extract on platelet aggregation: a randomized placebo-controlled double-blind study. *Clinical and Experimental Pharmacology and Physiology* **22**: 414-417.

Morrison, M.H., Dimonte, D. and Jernstrom, B., 1985. Glutathione status in primary cultures of rat hepatocytes and its role in cell attachment to collagen. *Chemco-Biological Interactions* **53**: 3-12.

Muller, D., Glockner, R., Rost, M. and Steinmetzer, P., 1998. Monooxygenation, cytochrome P450-mRNA expression and other functions in precision-cut rat liver slices. *Experimental and Toxicologic Pathology* **50**: 507-513.

Nagasaki, H., Nakano, H. and Boudjema, K., 1998. Efficacy of preconditioning with N-acetylcysteine against reperfusion injury after prolonged cold ischaemia in rat liver in which glutathione had been reduced by buthionine sulphoximine. *European Journal of Surgery* **164**: 139-146.

Nakagawa, S., Yoshida, S., Hirao, Y., Kasaga, S. and Fuwa, T., 1985. Cytoprotective activity of components of garlic, ginseng and ciuwjia on hepatocyte injury induced by carbon tetrachloride in vitro. *Hiroshima Journal of Medical Sciences* **34**: 303-309.

Nakagawa, S., Kasaga, S. and Matsuura, H., 1989. Prevention of liver damage by aged garlic extract. *Phytocherapy Research* **3**: 50-53.

Narashimhan, N., Weller, P.E., Buben, J.A., Wiley, R.A. and Hanzlik, R.P., 1988. Microsomal metabolism and covalent binding of [$^{3}H/^{14}C$] bromobenzene. Evidence for quinones as reactive metabolites. *Xenobiotica* **18**: 491-499.

Neil, H., Silagy, C.A., Lancaster, T., Hodgeman, J., Vos, K., Moore, J.W., Jones, L., Cahill, J. and Fowler, G.H., 1996. Garlic powder in the treatment of moderate hyperlipidaemea: a controlled trial and meta-analysis. *Journal of Royal College of Physicians of London* **30**: 329-334.

Nelson, D.R., Kamataki, T., Waxman, D.J., Guengerich, F.P., Estabrook, R.W., Feyereisen, R., Gonzalez, F.J., Coon, M.J., Gunsalus, I.C., Gotoh, O., Okuda, K. and Nebert, D.W., 1993. The P450 superfamily: uptake on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. *DNA and Cell Biology* **12**: 1-51.

Offen, D., Ziv, I., Sternin, H., Melamed, E. and Hochman, A. 1996. Prevention of dopamine-induced cell death by thiol antioxidants: possible implications for treatment of Parkinson's disease. *Experimental Neurology* **141**: 32-39.

Ogawa, H., Suezawa, K., Meguro, T. and Sasagawa, S., 1993. Effect of garlic powder on lipid metabolism in stroke-prone spontaneously hypertensive rats. *Nippo Eiyo, Shokyryo Gakkaishi* **46**: 417-423.

Ohkawa, H., Ohishi, N. and Yagi, K., 1979. Assay of lipid peroxides in animal tissue by thiobarbituric acid reaction. *Analytical Biochemistry* **95**: 351-358.

Olinga, P., Green, K., Hof, I.H., De Kanter, R., Koster, H.J., Leeman, W.R., Rutten, A.A.J.J.L., Van Twillert, K. and Groothuis, G.M.M., 1997. Comparison of five incubation systems for rat liver slices using functional and viability parameters. *Journal of Pharmacological and Toxicological Methods* **38**: 59-69.

Omura, T. and Sato, R., 1964. The carbon monoxide binding pigment of liver microsomes. *Journal Biological Chemistry* **239**: 2370-2378.

Oshio, C. and Phillips, M.J., 1981. Contractility of bile canaliculi; implications for liver function. *Science* **212**: 1041-1042.

Page, R.A., Stowell, K.M., Hardman, M.J. and Kitson, K.E., 1992. The assessment of viability in isolated rat hepatocytes. *Analytical Biochemistry* **200**: 171-175.

Paine, A.J. and Legg, R.F., 1978. Apparent lack of correlation between the loss of cytochrome P-450 in hepatic parenchymal cell culture and the stimulation of haem oxygenase. *Biochemical Biophysical Research Communications* **81**: 672-679.

Paine, A.J. and Hockin, L.J., 1980. Nutrient imbalance causes the loss of cytochrome P-450 in liver cell culture: formation of culture media which maintain cytochrome P-450 at in vivo concentrations. *Biochemical Pharmacology* **29**: 3215-3218.

Paine, A.J., 1990. The maintenance of cytochrome P450 in rat hepatocyte culture: some applications of liver cell cultures to the study of drug metabolism, toxicity and the induction of the P450 system. *Chemico-Biological Interactions* **74**: 1-31.

Park, B.K., Pirmohamed, M.P. and Kitferingham, N.R., 1992. Idiosyncratic drug reactions; a mechanistic evaluation of risk factors. *British Journal of Clinnical Pharmacology* **34**: 377-395.

Park, B.K., 1995. Acetaminophen-induced hepatotoxicity: protection by enzyme inhibition. *European Journal of Gastroenterology & Hepatology* **7**: 747-748.

Park, J.C., Yu, Y.B., Lee, J.H., Hattori, M., Lee, C.K. and Choi, J.W., 1996. Protective effect of oenanthe javanica on the hepatic lipid peroxidation in bromobenzene-treated rats and its bioactive component. *Planta Medica* **62**: 488-480.

Park, K.A. and Choi, H., 1997. Modification of hepatic microsomal cytochrome P-450 2E1 enzyme by garlic powder in rat hepatocarcinogenesis. *Journal Biochemistry Molecular Biology* **30**: 73-79.

Pinto, J.T., Qiao, C., Xing, J., Rivlin, R.S., Protomastro, M.L., Weissler, M.L., Tao, Y., Thaler, H. and Heston, W.D.W., 1997. Effects of garlic thioallyl derivations on growth, glutathione concentration, and polyamine formation of human prostate carcinoma cells in culture. *American Journal Clinical Nutrition* **66**: 398-405.

Polasa, K. and Krishnaswamy, K., 1997. Reduction of urinary mutagen excretion in rats fed garlic. *Cancer Letters* **114**: 185-186.

Pollard, M.R. and Dutton, G.J., 1982. Liver snips; a simple, rapid and reproducible method for studying metabolism in small fragments of tissue, as applied to glucuronidation in rat liver. *Biochemical Journal* **202**: 469-473.

Pomerat, C.M. and Leake, C.D., 1954. Short term cultures for drug assays: general considerations. *Annals. New York Academy of Sciences* **58**: 1110-1128.

Prasad, K., Laxdal, V.A, Yu, M. and Raney, B.L., 1996. Evaluation of hydroxyl radical-scavenging property of garlic. *Molecular and Cellular Biochemistry* **154**: 55-63.

Prescott, L.F. and Critchely, J.A., 1983. The treatment of acetaminophen poisoning. *Annual Review of Pharmacology and Toxicology* **23**: 87-101.

Price, R.J., Mistry, H., Wield, P.T., Renwick, A.B., Beamand, J.A. and Lake, B.G., 1996. Comparison of the toxicity of allyl alcohol, coumarin and menadione in precision-cut rat, guinea-pig, Cynomolgus monkey and human liver slices. *Archives of Toxicology* **71**: 107-111.

Pryor, W.A. and Castle, L., 1984. Chemical methods for the detection of lipid hydroperoxides. *Methods in Enzymology* **105**: 293-299.

Push, S., 1984. Scientists eye draize test alternatives. *The Johns Hopkins Center for Alternative to Animal Testing Newsletter.* **3**: 1-8.

Pushpendran, C.K., Devasagayam, J.P. and Eapen, J., 1982. Age related effect of diallyl disulphide in rats. *Indian Journal of Experimental Biology* **20**: 428-429.

Quistorff, B., Bondesen, S. and Grunnet, N., 1973. Preparation and biochemical charaterization of parenchymal cells from rat liver. *Biochemia et Biophysica Acta* **320**: 503-516.

Rafeiro, E., Barr, S.G., Harrison, J.J. and Racz, W.J., 1994. Effects of N-acetylcysteine and dithiothreitol on glutathione and protein thiol replenishment during acetaminophen-induced toxicity in isolated mouse hepatocytes. *Toxicology* **93**: 209-224.

Ramos, K., Combs, A.B. and Acosta, D., 1983. Cytotoxicity of isoproterenol to cultured heart cells: effects of antioxidants on modifying membrane damage. *Toxicology and Applied Pharmacology* **70**: 317-323.

Ravindranath, V. and Boyd, M.R., 1991. Effect of modulators of glutathione synthesis of the hepatotoxicity of 2-methylfuran. *Biochemical Pharmacology* **41**: 1311-1318.

Rauckman, E.J. and Padilla, G.M., 1987. The Isolated Hepatocyte: Use in Toxicology and Xenobiotic Biotransformations, (E.J.Rauckman and G.M.Padilla, eds.), Academic Press, Inc., Orlando, FL.

Redman, C.M., Banerjee, D., Howell, K. and Palade, G.E., 1975. Colchicine inhibition of plasma protein release from rat hepatocytes. *Journal of Cell Biology* **61**: 42-59.

Reed, D.J. and Orrenius, S., 1977. The role of methionine in glutathione biosynthesis by isolated hepatocytes. *Biochemical and Biophysical Research Communications* **77**: 1257-1264.

Reicks, M.M. and Crankshaw, D.L., 1996. Modulation of rat hepatic cytochrome P-450 activity by garlic organosulphur compounds. *Nutrition and Cancer* **25**: 241-248.

Reid, W.D., Christie, B., Krishna, G., Michell, J.R., Moskowitz, J. and Brodie, B.B., 1971. Bromobenzene metabolism and hepatic necrosis. *Pharmacology* **6**: 47-55.

Remmer, H., 1972. Induction of drug metabolizing enzyme system in the liver. *European Journal of Clinical Pharmacology* **5**: 116-118.

Reuter, H.D., Koch, H.P. and Lawson, L.D., 1996. Therapeutic effects and applications of garlic and its preparations. In: Garlic. The Science and Therapeutic Application of *Allium Sativum L.* and Related Species, (H.P.Koch and L.D.Lawson, eds.), Williams and Wilkins, 2rd eddition.

Reynolds, E.S., 1963. The use of lead citrate at high pH as an electron-opague stain in electron microscopy. *Journal of Cell Biology* **17**: 208-212.

Richards, P.S. and Saba, T.M., 1985. Effect of endotoxin on fibronectin and Kupffer cell activity. *Hepatology* **5**: 32-37.

Rogiers, V., Adriaenssens, L., Vandenberghe, Y., Gepts, E., Callaerts, A. and Vercruysse, A., 1986. Critical evaluation of 7-ethoxycoumarin O-deethylase activity measurement in intact isolated rat hepatocytes. *Xenobiotica* **16**: 817-826.

Russo, M.A., Van Rossum, G.D.V. and Galestti, T., 1977. Observations on the regulation of cell volume and metabolic control in vitro. Changes in the composition and ultrastructure of liver slices under conditions or varying metabolic and transporting activity. *Journal of Membrane Biology* **31**: 267-299.

Salyers, K.L., Barr, J. and Sipes, I.G., 1994. In vitro metabolism of theophylline by rat and human liver slices. *Xenobiotica* **24**: 389-399.

Santone, K.S. and Acosta, D., 1984. The role of extracellular calcium in CCl_4 injury of cultured rat hepatocytes. *Toxicologist* **4**: 133-137.

Schaffer, E.M., Liu, J.Z, Green, J., Dangler, C.A. and Milner, J.A., 1996. Garlic and associated allyl sulfur components inhibit N-methyl-N-nitrosourea induced rat mammary carcinogenesis. *Cancer Letters* **102**: 199-204.

Schaffer, E.M., Liu, J.Z. and Milner, J.A., 1997. Garlic powder and allyl sulfur compounds enhance the ability selenite to inhibit 7,12-dimethylbenz[a]anthracene-induced mammary DNA adducts. *Nutrition and Cancer* **27(2)**: 162-168.

Scharfenberg, K., Ryll, T., Wagner, R. and Wagner, K.G., 1994. Injuries to cultivated BJA-B cells by ajoene, a garlic-derived natural compound: cell viability, glutathione metabolism and pools of acidic amino acids. *Journal Cellular Physiology* **158**: 55-60.

Scott, G., Emerton, D.A.N. and Swain, C.M., 1995. Influence of various cold storage solutions and culture media on selected drug metabolism activities of rat liver slices. *ISSX Proceedings* **8**: 417.

Sedlak, J. and Lindsay, R.H., 1968. Estimation of total, protein-bound and nonprotein sulfhydryl groups in tissue with Ellman's reagent. *Analytical Biochemistry* **25**: 192-205.

Segal, H.L. and Matsuzawa, T., 1970. L-alanine aminotrasferase (rat liver). *Methods in Enzymology* **17A**: 153-159.

Shoji, S., Furuishi, K., Yanase, R., Miyazaka, T. and Kino, M., 1993. Allyl compounds selectively killed human immunodeficiency virus (type 1)-infected cells. *Biochemical and Biophysical Research Communications* **194**: 610-621.

Sidelmann, U.G., Cornett, C., Tjqrnelund, J. and Hansen, S.H., 1996. A comparative study of precision cut liver slices, hepatocytes, and liver microsomes from the Wistar rat using metronidazole as a model substance. *Xenobiotica* **26**: 709-722.

Sigounas, G., Hooker, J., Anagnostou, A. and Steiner, M., 1997. S-allylmercaptocysteine inhibits cell proliferation and reduces the viability of erythroleukemia, breast and prostate cancer cell lines. *Nutrition and Cancer* **27(2)**: 186-191.

Simko, V., Michael, S., Katz, J., Oberstein, E. and Popescu, A., 1992. Protective effect of oral acetylcysteine against the hepatorenal toxicity of carbon tetrachloride potentiated by ethyl alcohol. *Alcoholism: Clinical and Experimental Research* **16**: 795-799.

Singh, A. and Rao, A.R., 1995. Evaluation of the modifying influence of acecanut on the garlic-modulated hepatic detoxication system. Enzymes, sulfhydryl content, and lipid peroxidation in mice. *Teratogenesis, Carcinogenesis, and Mutagenesis* **15**: 127-134.

Singh, S.P., Abraham, S.K. and Kesavan, P.C., 1995. In vitro radioprotection with garlic extract. *Mutation Research* **345**: 147-153.

Singh, Y., Cooke, J.B., Hinton, D.E. and Miller, G., 1996. Trout liver slices for metabolism and toxicity studies. *Drug Metabolism and Disposition* **24**: 7-14.

Sipes, I.G. and Gandolfi, A.J., 1982. Bioactivation of aliphatic organohalogens: formation, detection, relevance. In: Toxicology of the Liver, (G.L.Plaa and W.R.Hewitt, eds.), Raven Press, New York, pp. 181-212.

Slaughter, D.E. and Hanzlik, R.P., 1991. Identification of epoxide- and quinone-derived bromobenzene adducts to protein sulfur mucleophiles. *Chem. Res. Toxicol.* **4**: 349-359.

Smith, M.T., Thor, H., Hartzell, P. and Orrenius, S., 1982. The measurement of lipid peroxidation in isolated hepatocytes. *Biochemical Pharmacology* **31**: 19-26.

Smith, P.F., Gandolfi, A.J., Krumdieck, C.L., Putnam, C.W., Zukoski, C.F., Davis, W.M. and Brendel, K., 1985. Dynamic organ culture of precision liver slices for in vitro toxicology. *Life Sciences* **36**: 1367-1375.

Smith, P.F., Krack, G., McKee, R.L., Johnson, D.G., Gandolfi, A.J., Hruby, V.J., Krumdieck, C.L. and Brendel, K., 1986. Maintenance of adult rat liver slices in dynamic organ culture. *In Vitro Cellular & Developmental Biology* **22**: 706-712.

Smith, P.F., Fisher, R., Shubat, P.J., Gandolfi, A.J., Krumdieck, C.L. and Brendel, K., 1987. In vitro cytotoxicity of allyl alcohol and bromobenzene in novel organ culture system. *Toxicology and Applied Pharmacology* **87**: 509-522.

Smith, P.F., Fisher, R., McKee, R., Gandolfi, A.J., Krumdieck, C.L. and Brendel, K., 1988. Precision cut liver slices: a new in vitro tool in toxicology. In: In Vitro Toxicology: Model Systems and Methods, (C.McQeen, ed.), Telford Press, Inc., New Jersey, pp. 93-130.

Sorrentino, D., Ness, V., Ribeiro, K. and Miller, C.M., 1991. Functional and morphological features of isolated hepatocytes preserved in University of Wisconsin Solution. *Hepatology* **14**: 331-339.

Sparnins, V.L., Barang, G. and Wattenberg, L.W., 1988. Effects of organosulfur compounds from garlic and onions on benzo[a]pyrene-induced neoplasia and glutathione S-transferase activity in the mouse. *Carcinogenesis* **9**: 131-134.

Stacey, N.H., Cantilena, L.R. and Klaassen, C.D., 1980. Cadmium toxicity and lipid peroxidation in isolated rat hepatocytes. *Toxicology Applied Pharmacology* **53**: 470-480.

Stacey, N.H. and Klaassen, C.D., 1981. Comparison of the effects of metals on cellular injury and lipid peroxidation in isolated rat hepatocytes. *Journal of Toxicology and Environmental Health* **7**: 139-147.

Steiner, M., Khan, A.H. and Lin, R.I.S., 1996. A double blind crossover study in moderately hypercholesterolemic men comparing the effect of aged garlic extract and placebo administration on blood lipid and platelet function. *Shinyaku To Rinsho (New Drug Clin.)* **45(3)**: 456-466.

Stolzenbach, F., 1966. Lactic dehydrogenase (Crystalline). *Methods in Enzymology* **9**: 278-288.

Story, D.L., Gee, S.J., Tyson, C.A. and Gould, D.H., 1983. Response of isolated hepatocytes to organic and inorganic cytotoxins. *Journal Toxicology Environmental Health* **11**: 483-501.

Sturdee, A.P., Beirne, J.M., Sorman, A.E., Orton, T.C. and Grisp, D.M., 1983. Metabolic capability of rat hepatocytes isolated by perfusion and tissue slice techniques. *Life Sciences* **32**: 1463-1469.

Suarez, K.A., Bhonsle, P. and Richardson, D.L., 1986. Protective effect of N-acetycysteine pretreatment against cocaine induced hepatotoxicity and lipid peroxidation in the mouse. *Research Communications in Substances of Abuse* **7**: 7-18.

Sumiyoshi, H. and Wargovich, M.J., 1989. Garlic (*Allium sativum*): a review of its relationship to cancer. *Asia Pacific Journal of Pharmacology* **4**: 133-140.

Sundaram, S.G. and Milner, J.A., 1996. Diallyl disulfide suppresses the growth of human colon tumor cell xenografts in athymic nude mice. *Journal of Nutrition* **36**: 1355-1361.

Tanaka, K., Sato, M., Tomita, Y. and Ichihara, A., 1978. Biochemical studies on liver functions in primary cultured hepatocytes of adult rats. I. Hormonal effects on cell viability and protein synthesis. *Journal of Biochemistry* **84**: 937-946.

Tappel, A.L., 1980. Free Radicals in Biology, (W.A.Pryor, ed.), Academic Press, London, **IV**: 1-47.

Tarauchi, T. and Hirata, M., 1994. Effects of P-450 induces on glutathione (GSH) depletion by bromobenzene in primary cultures of dog hepatocytes. *Biological & Pharmceutical Bulletin* **17**: 658-661.

Tavoloni, N. and Berk, P.D., 1993. Hepatic Transport and Bile Secretion: Physiology and Pathophysiology. 2nd. ed., Raven Press, Ltd.

Tephly, T.R. and Burchell, B., 1990. UDP glucuronyl transferases: a family of detoxication enzymes. *Trends Pharmacological Sciences* **11**: 267-279.

Thibodean, G.A. and Patton, K.T., 1993. Anatomy and Physiology. 2nd. ed., Mosby-Year Book, Inc., U.S.A.

Thor, H., Moldeus, P., Hermanson, R., Hogberg, J., Reed, D.J. and Orrenius, S., 1978. Metabolic activation and hepatotoxicity. Toxicity of bromobenzene in hepatocyte isolated from phenobarbital- and diethylmaleate-treated rats. *Archives Biochemistry Biophysics* **188**: 122-129.

Thor, H., Moldeus, P. and Orrenius, S., 1979. Effect of cysteine, N-acetylcysteine and methionine on glutathione biosynthesis and bromobenzene toxicity in isolated rat hepatocytes. *Archives Biochemistry Biophysics* **192**: 405-413.
Thor, H. and Orrenius, S., 1980. The mechanism of bromobenzene-induced cytotoxicity studied with isolated hepatocytes. *Archives of Toxicology* **44**: 31-43.

Thurman, R.G. and Kauffman, F.C., 1980. Factors regulating drug metabolism in intact hepatocytes. *Pharmacological Review* **31**: 229-251.

Thurman, R.G., Ganey, P.E., Belinsky, S.A., Conway, J.G., Badr, M.Z. and Kauffman, F.C., 1988. Advantages of the perfused liver as a model to study hepatotoxicity in periportal and pericentral regions of the liver lobule. In: In Vitro Toxicology: Model Systems and Methods, (C.A.McQeen, ed.), Telford Press, Inc., New York, pp. 69-92.

Timbrell, J.A., 1991. Principles of Biochemical Toxicology. 2nd, ed., Taylor & Francis Ltd., London, Washington DC.

Togashi, H., Shinzawa, H., Wakabayshi, H., Nakamura, T., Youg, H., Yamada, N., Ukai, K., Okuyama, Y., Takahashi, T. and Ishikawa, M., 1991. Superoxide is involved in the pathogenesis of paraquat-induced injury in cultured rat-liver slices. *Hepatology* **14**: 707-714.

Trams, E.G. and Lauter, C.J., 1974. On the sideness of plasma membrane enzymes. *Biochimia et Biophysica Acta* **345**: 180-197.

Trowell, O.A., 1959. The culture of mature organs in a synthetic medium. *Experimental Cell Research* **16**: 118-147.

Tyson, C.A. and Green, C.E., 1987. Cytotoxicity measures: choices and methods. In: The Isolated Hepatocyte: use in toxicology and xenobiotic biotransformations, (E.J.Rauckman & G.M.Padilla, eds.), Academic Press, Inc., pp. 119-158.

Urbina, J.A., Marchan, E., Lazardi, K., Visbal, G., Apitz-Castro, R., Gil, R., Aguirre, F., Piras, T., M.M. and Piras, R., 1993. Inhibition of phosphatidylcholine biosynthesis and cell proliferation in trypanosoma cruzi by ajoene, an antiplatelet compound isolated from garlic. *Biochemical Pharmacology* **45**: 2381-2387.

Uwaifo, A.O., 1984. Inhibition of oxygen-uptake in liver slices of 3 mammalian-species (rat, rabbit, guinea-pig) by aflatoxin-B_1 (AFB_1). *Toxicology* **31**: 33-39.

Van Rossum, G.D.V. and Russo, M.A., 1981. Ouabain-resistant mechanism of volume control and the ultra-structural organization of liver slices recovering from swelling in vitro. *Journal Membrane Biology* **59**: 191-209.

Wang, E.J., Li, Y., Lin, M., Chen, L., Stein, A.P., Reuhl, K.R. and Yang, C.S., 1996. Protective effects of garlic and related organosulphur compounds on acetaminophen-induced hepatotoxicity in mice. *Toxicology and Applied Pharmacology* **136**: 146-154.

Waymouth, C., 1972. Construction of tissue culture media. In: Growth, Nutrition and Metabolism of Cells in Culture, (G.H.Rothblat and V.J.Gristofalo, eds.), New York and London, Academic Press, **1**: 11-47.

Waymouth, C., 1974. Feeding the baby-designing the culture milieu to enhance cell stability. *Journal of the National Cancer Institute* **53**: 1443-1448.

Welch, C., Wuarin, L. and Sidell, N., 1992. Antiproliferative effect of the garlic compound S-allyl cysteine on human neuroblastoma cell in vitro. *Cancer Letters* **63**: 211-219.

Weller, P.A., Narashimhan, N., Buben, J.A. and Hanzlik, R.P., 1988. In vitro metabolism and covalent binding among ortho-substituted bromobenzene of varying hepatotoxicity. *Drug Metabolism and Disposition* **16**: 232-237.

Williams, R.T., 1959. Detoxication Mechanisims. 2nd, edition, London: Chapman & Hall.

Winterbourne, D.J., 1993. Chemical assays for proteins. In: Methods in Molecular Biology. 19: Biomembrane Protocols: 1. Isolation and Analysis, (J.M.Graham and J.A.Higgins, eds.), Human Press Inc., 197-202.

Wishnies, S.M., Parrish, A.R., Sipes, I.G., Gandolfi, A.J., Putnam, C.L., Krumdieck, C.L. and Brendel, K., 1991. Biotransformation activity in vitrified human liver slices. *Cryobiology* **28**: 216-226, 1991.

Wolf, S. and Reim, M., 1990. Effect of garlic on conjunctival vessels: a randomised, placebo-controlled, double-blined trial. *British Journal of Clinical Practices* **44**: 36-39.

Wolfgang, G.H.I., Donarski, W.J. and Petry, T.W., 1990a. Effects of novel antioxidants on carbon tetrachloride-induced lipid peroxidation and toxicity in precision-cut rat liver slices. *Toxicology and Applied Pharmacology* **106**: 63-70.

Wolfgang, G.H.I., Gandolfi, A.J., Nagle, R., Brendel, K. and Stevens, J., 1990b. Assessment of S-(1,2-dichlorovinyl)-L-cysteine induced toxic events in rabbit renal cortical slices. Biochemical and histological evaluation of uptake, covalent binding, and toxicity. *Chemico-Biological Interactions* **75**: 153.

Wolfgang, G.H.I., Jolly, R.A., Donarski, W.J. and Petry, T.W., 1991. Inhibition of diquat-induced lipid peroxidation and toxicity in precision-cut liver slices by novel antioxidants. *Toxicology and Applied Pharmacology* **108**: 321-329.

Wong, B.K., Chan, H.C. and Corcoran, G.B., 1986. Selective effects of N-acetylcysteine stereoisomers on hepatic glutathione and plasma sulfate in mice. *Toxicology and Applied Pharmacology* **86**: 421-429.

Worboys, P.D., Bradbury, A. and Houston, B.J., 1997. Kinetics of drug metabolism in rat liver slices. III. Relationship between metabolic clearance and slice uptake rate. *Drug Metabolism and Disposition* **25**: 460-467.

Wright, M.C. and Paine, A.J., 1992. Evidence that the loss of rat liver cytochrome P450 in vitro is not solely associated with the use of collagenase, the loss of cell-cell contacts and /or the absence of an extracellular matrix. *Biochemical Pharmacology* **43(2)**: 237-243.

Wu, J. Danielsson, A., Lindstrom, P., Karlsson, K. and Sehlin, J., 1995. Protective effects of calcium channel blockers on acute bromobenzene toxicity to isolated rat hepatocytes. *Scandinavian Journal of Gastroenterology* **30**: 590-600.

Wu, J, Karlsson, K. and Danielsson, A., 1996. Protective effects of trolox C, vitamin C, and catalase on bromobenzene-induced damage to rat hepatocytes. *Scadinavian Journal of Gastroeneterology* **31**: 797-803.

Yagi, K., 1982. Lipid Peroxides in Biology and Medicine, (K.Yagi, ed.), Academic Press, New York, 223-243.

Yamasaki, T., Li, L. and Lau, H.S., 1994. Garlic compounds protect vascular endothelial cells from hydrogen peroxide-induced oxidation injury. *Phytotherapy Research* **8**: 408-412.

Yao, W.B., Zhao, Y.Q., Abe, T., Ohta, J. and Ubuka, T., 1994. Effects of N-acetyl cysteine administration on cysteine and glutathione contents in liver and kidney and in perfused liver of intact and diethyl maleate-treated rats. *Amino Acids* **7**: 255-266.

Yeh, Y.Y. and Yeh, S.M., 1994. Garlic reduces plasma lipids by inhibiting hepatic cholesterol and triacyglycerol synthesis. *LIPIDS* **9**: 189-193.

Zimmerman, H.J., Kendler, J., Libber, S. and Lukacs, L., 1974. Hepatocyte suspensions as a model for demonstration of drug hepatotoxicity. *Biochemical Pharmacology* **3**, 2187-2189.

Zimmerman, H.J., 1978. Hepatotoxicity. The Adverse Effects of Drugs and other Chemicals on the Liver, (H.J.Zimmerman, ed.), Appleton-Century-Crofts/New York, 169-180.

APPENDIX I

TYPICAL STANDARD CURVES FOR BIOCHEMICAL ASSAYS

1. Standard Curve of the Protein Assay by Coomassie Blue Staining Method

2. Standard Curve of the Spectrophotometric TBARS Assay

3. Standard Curve of the Flame-Photometric Potassium Assay

4. Standard Curve of the Bioluminometric ATP Assay

5. Standard Curve of the Spectrophotometric NPSH Assay

6. Standard Curve of the Spectrophotometric GSH Assay by DTNB-GSSG Reductase Recycling Method

7. Standard Curve of the Fluorometric ECOD Assay

8. Standard Curve of the Fluorometric PROD Assay

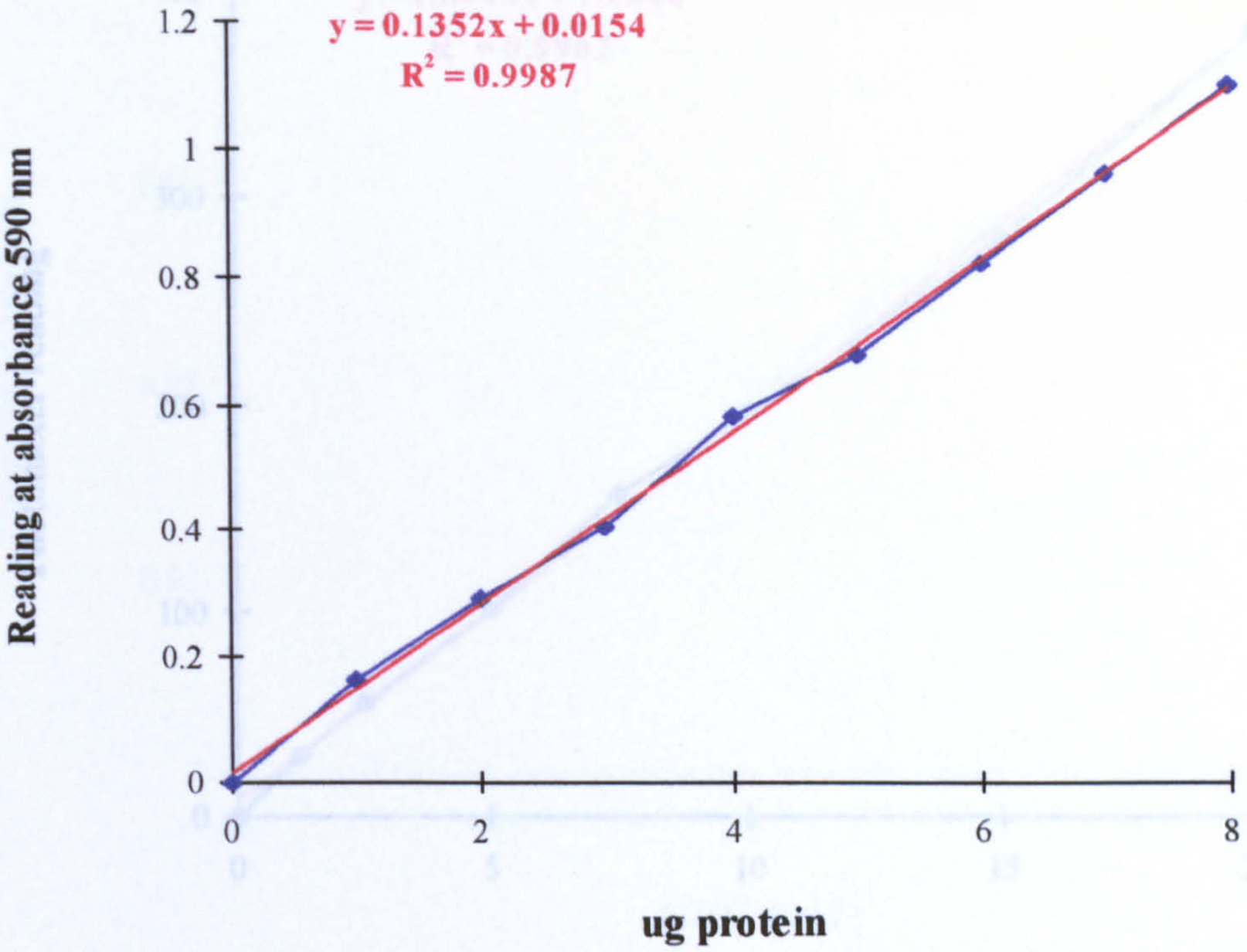

Figure 1. Standard curve of the protein assay by Coomassie blue staining method

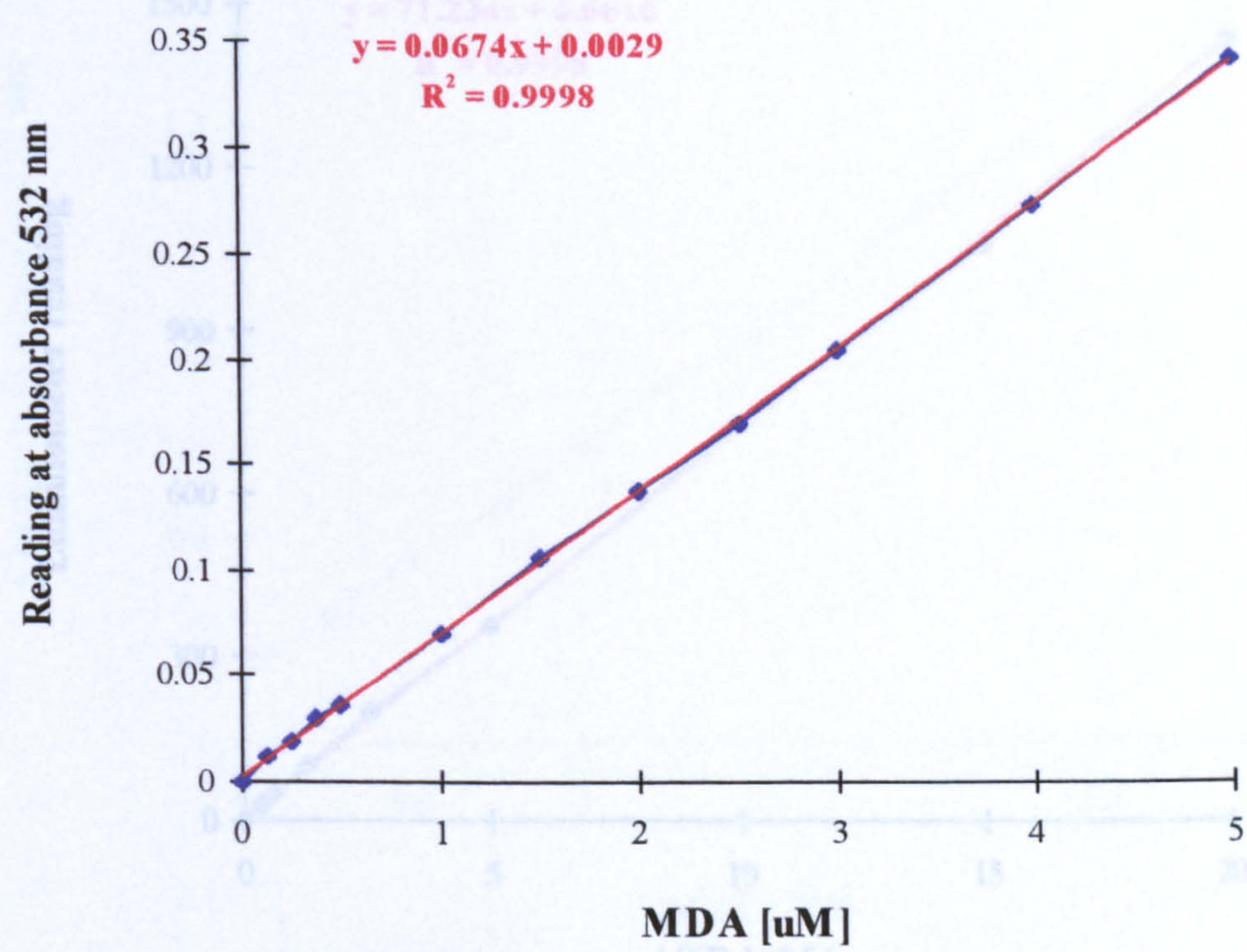

Figure 2. Standard curve of the spectrophotometric TBARS assay

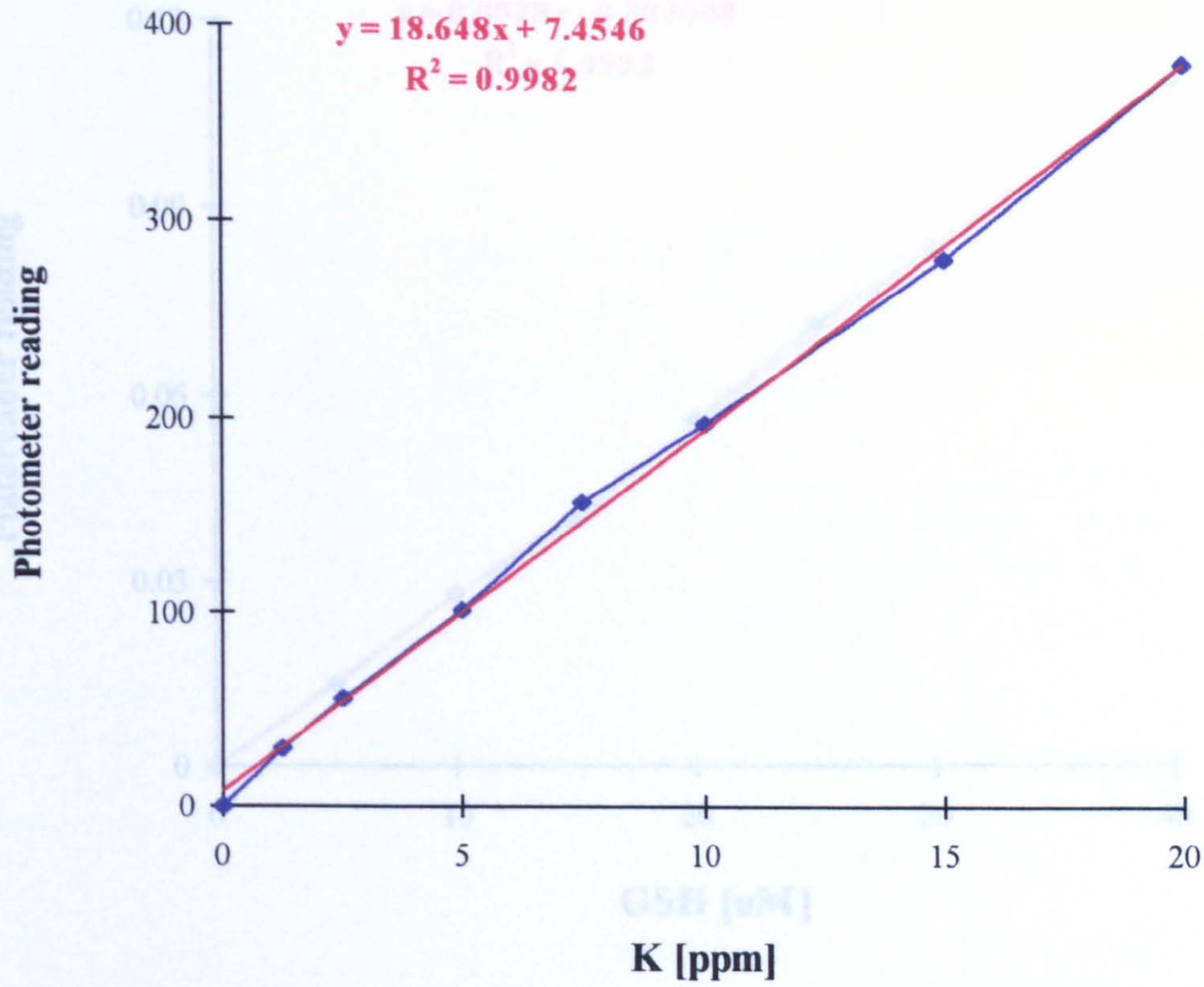

Figure 3. Standard curve of the flame-photometric potassium assay

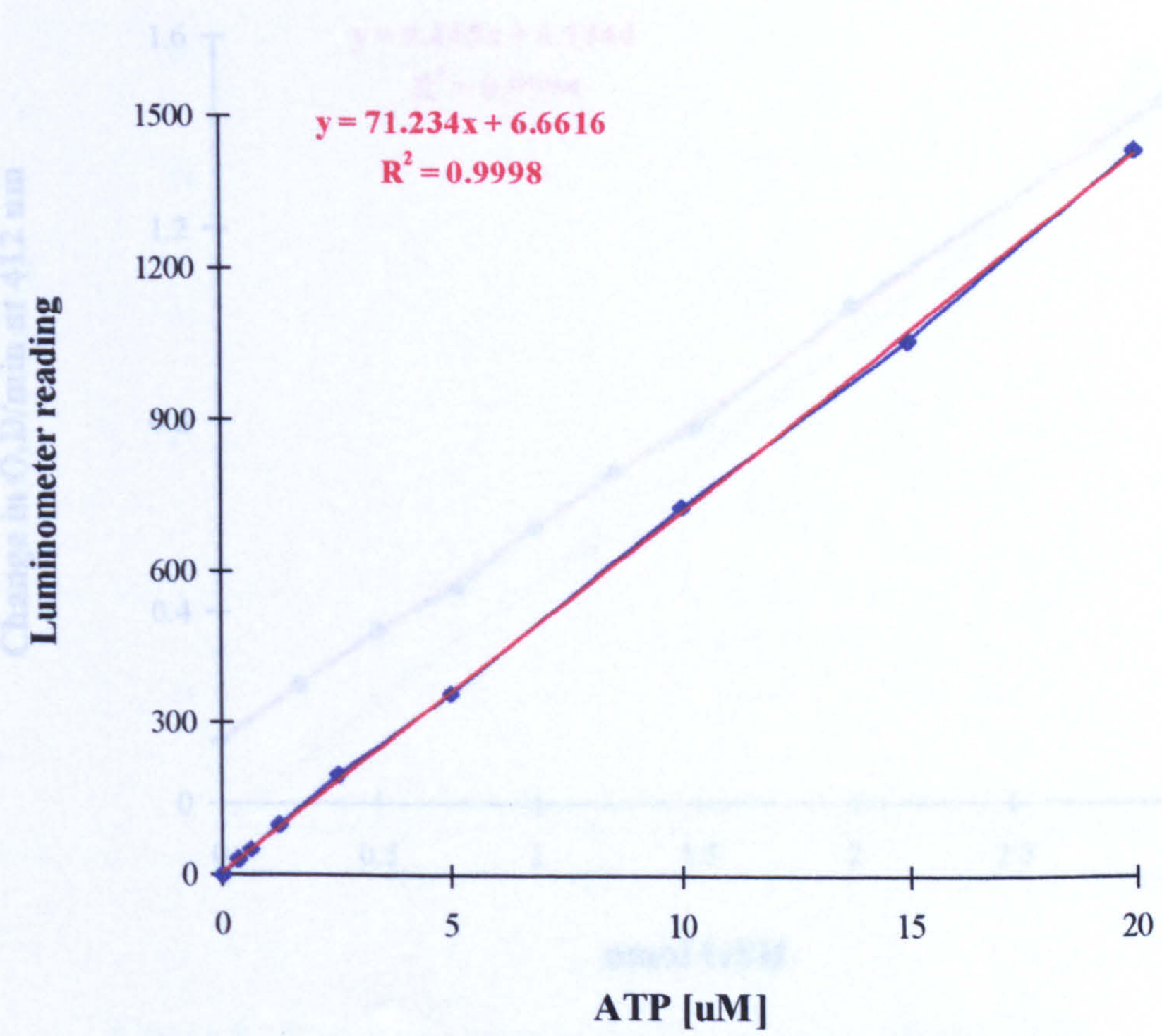

Figure 4. Standard curve of the bioluminometric ATP assay

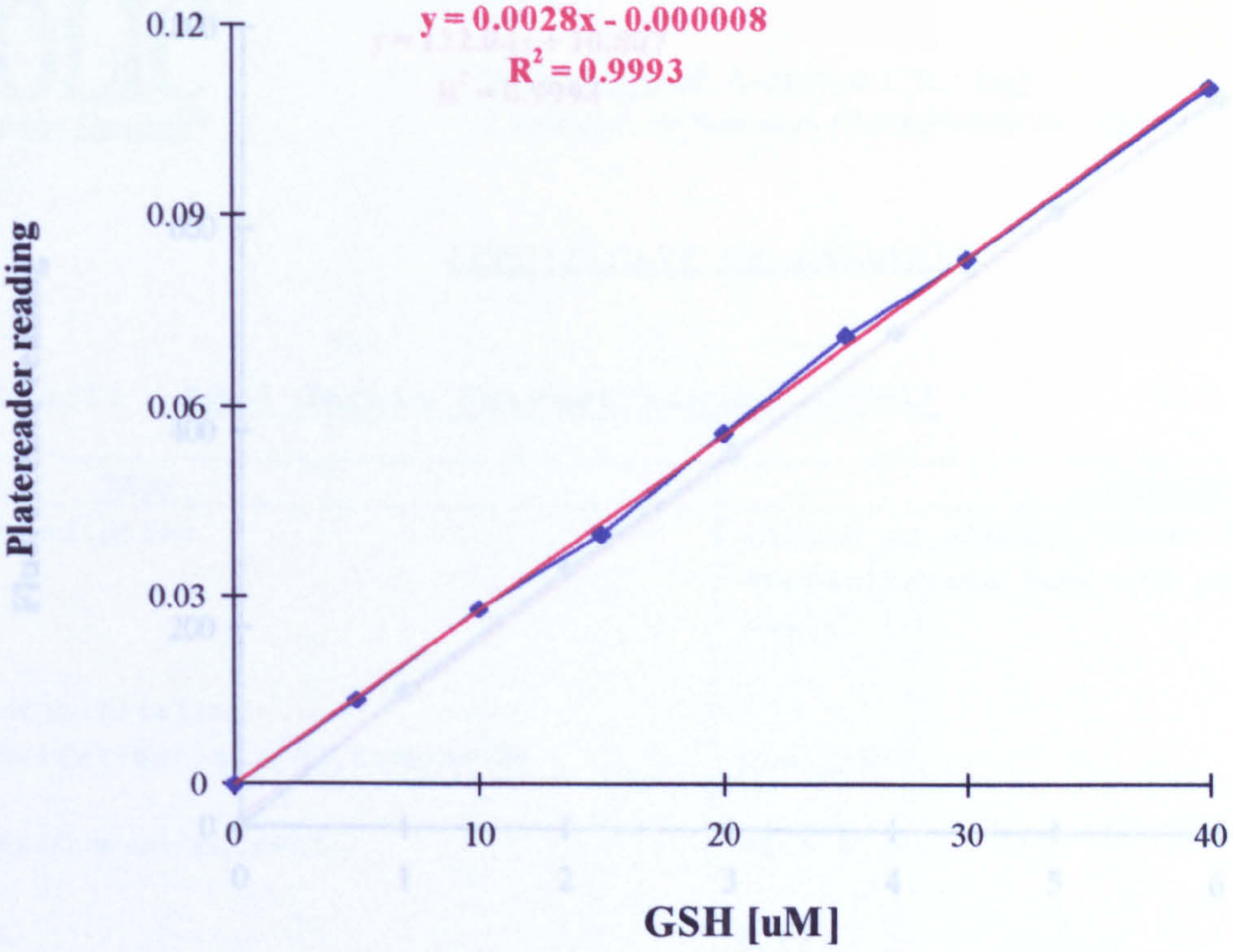

Figure 5. Standard curve of the spectrophotometric NPSH assay

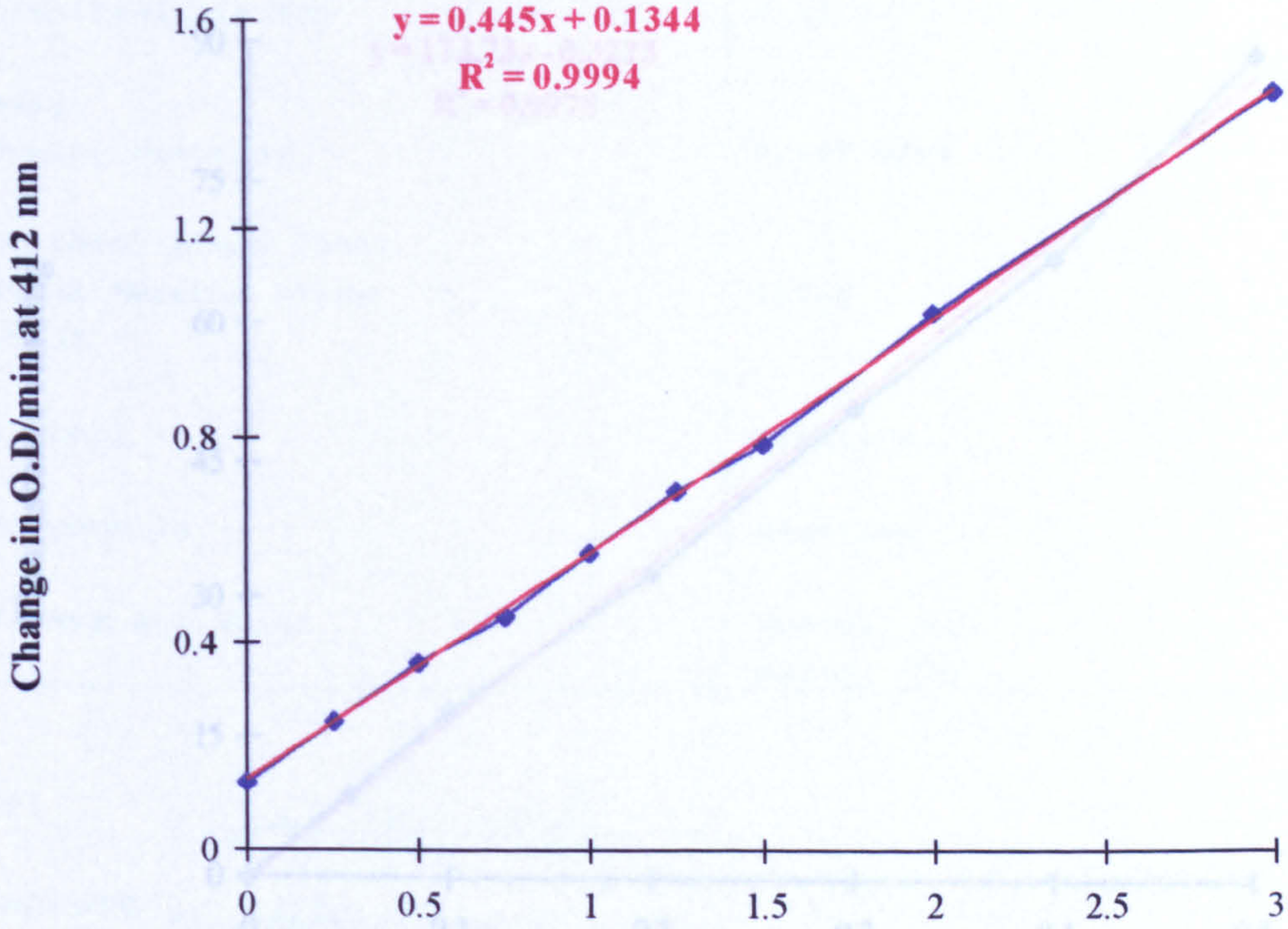

Figure 6. Standard curve of the spectrophotometric GSH assay by DTNB-GSSG reductase recycling method

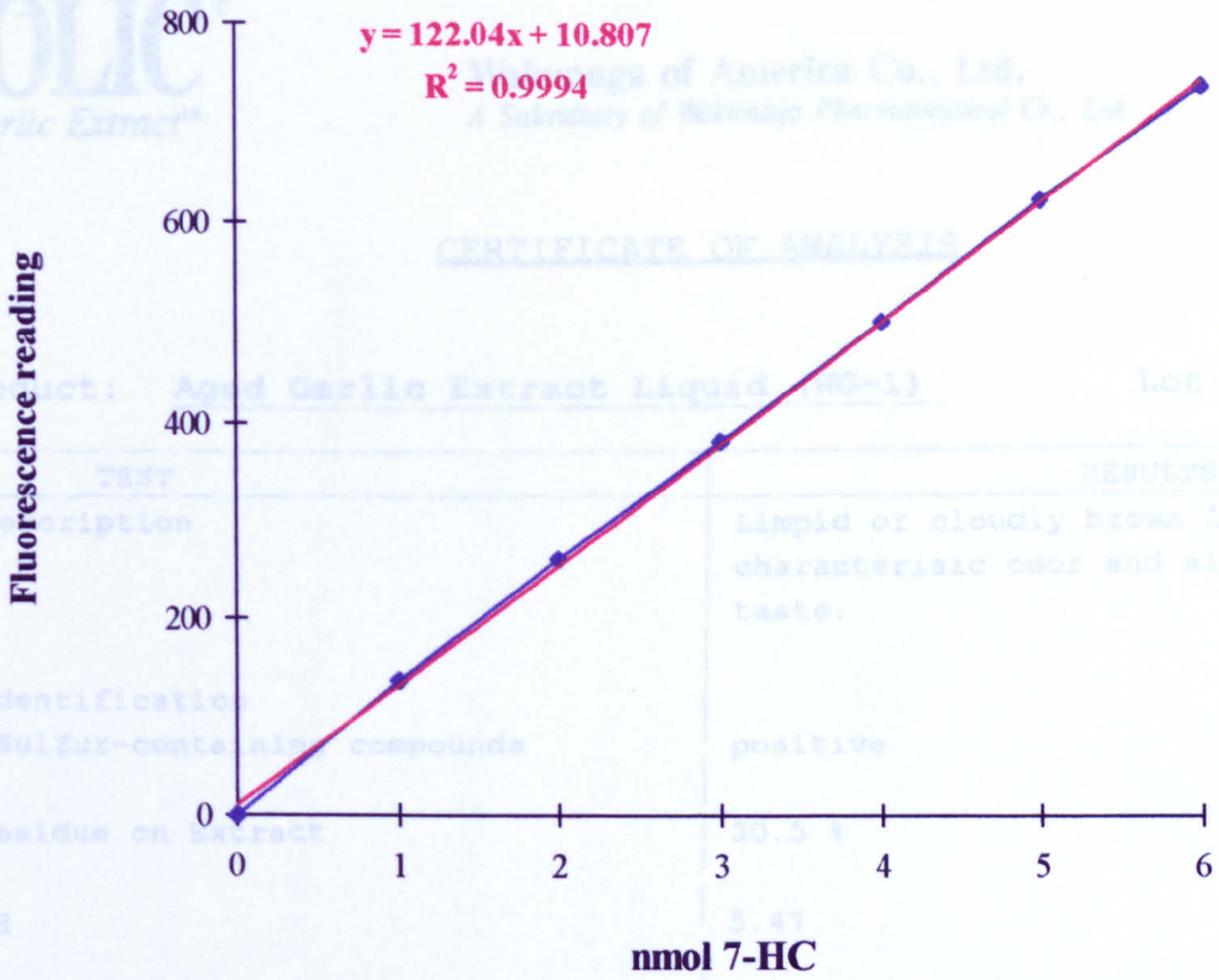

Figure 7. Standard curve of the fluorometric ECOD assay

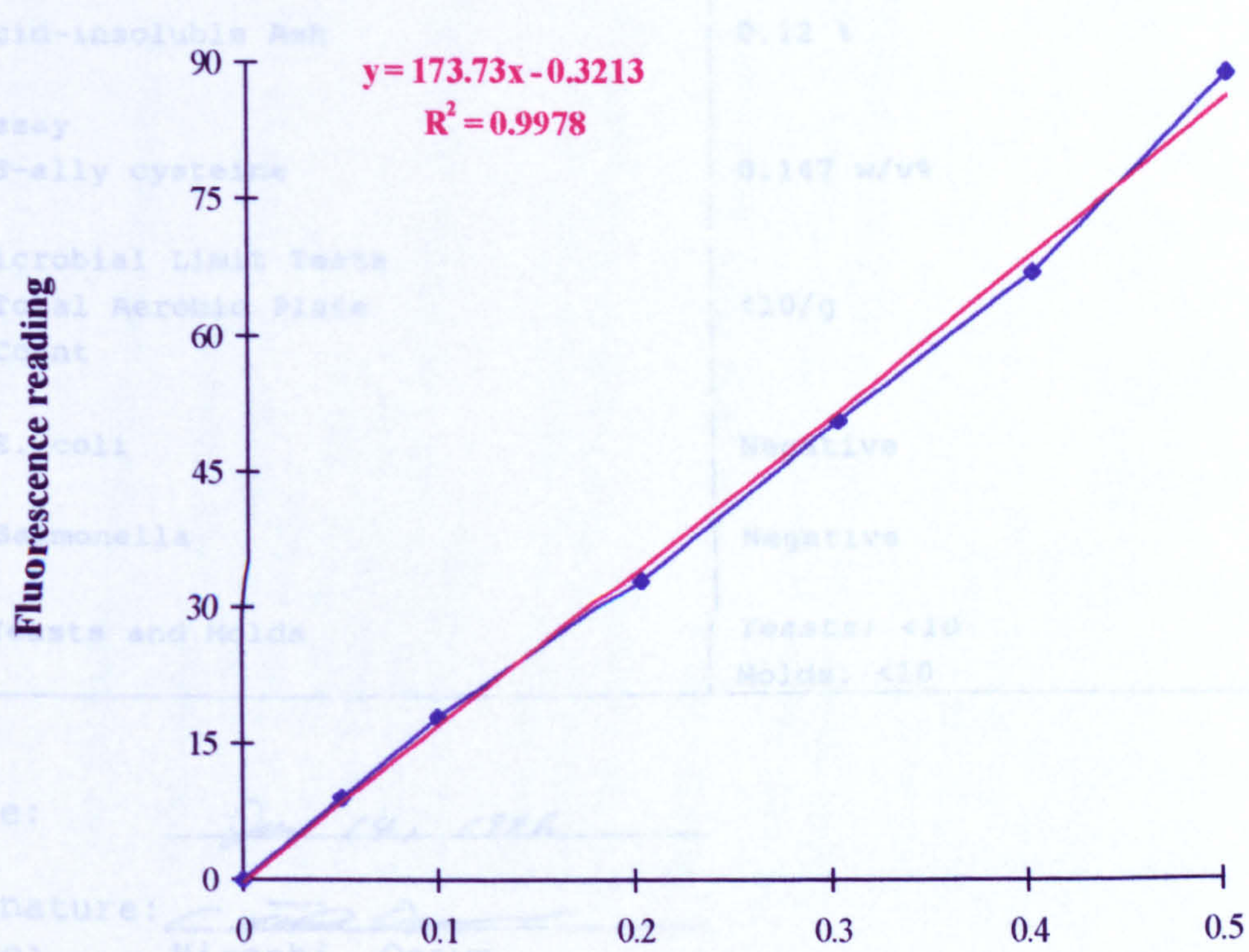

Figure 8. Standard curve of the fluorometric PROD assay

KYOLIC®
Aged Garlic Extract™

Wakunaga of America Co., Ltd.
A Subsidiary of Wakunaga Pharmaceutical Co., Ltd.

CERTIFICATE OF ANALYSIS

Product: **Aged Garlic Extract Liquid (WG-1)** Lot No.: 6E0:

TEST	RESULTS
* Description	Limpid or cloudly brown liquid with the characterisic odor and slightly sweet taste.
* *Identification* Sulfur-containing compounds	positive
* Residue on Extract	30.5 %
* pH	5.47
* Heavy Metals	<20 ppm
* Total Ash	1.03 %
* Acid-insoluble Ash	0.12 %
* Assay S-ally cysteine	**0.147 w/v%**
* Microbial Limit Tests Total Aerobic Plate Count	<10/g
E. coli	Negative
Salmonella	Negative
Yeasts and Molds	Yeasts: <10 Molds: <10

Date: June 14, 1996

Signature:
Name: Hiroshi Ogawa
Title: Manager, Quality Control
Wakunaga of America Co., Ltd.

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