STABILITY AND TRANSNITROSATION EFFICACY OF S-NITROSOTHIOLS IN BIOLOGICAL MODEL SYSTEMS

by

Ahmed Hussein Hussein Al-Mustafa

M.S. Biological Science,

Yarmouk University, Irbid, Jordan, 1993

B.S. Nutrition,

Yarmouk University, Irbid, Jordan, 1987

Submitted to the Mathematisch-Naturwissenschaftliche Fakultät of Heinrich-Heine-Universität Düsseldorf in partial fulfilment of the requirements for the degree of

Dr. rer. nat.

in

Biology

Düsseldorf 2001

STABILITY AND TRANSNITROSATION EFFICACY OF S-NITROSOTHIOLS IN BIOLOGICAL MODEL SYSTEMS

Gedruckt mit der Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Heinrich-Heine-Universität Düsseldorf

Referent: **Prof. Dr. Dr. h.c. Helmut Sies**

Korreferent: **Prof. Dr. Frank Wunderlich**

Tag der mündlichen Prüfung: 25 Oktober 2001

Table of contents

List of Figures

List of Tables

Abbreviations

CAPS: 3-cyclohexylamino-1-propanesulfonic acid

CDNB: 1-Chloro-2,4-dinitrobenzene

CGNO: S-nitrosocysteinylglycine

cGST: Cytosolic glutathione S-transferases

CysNO: S-Nitrosocysteine

DEA: Diethanolamine

EDTA: Ethylene diamine tetraacetic acid

GSH: Glutathione

GSNO: S-nitrosoglutathione

HCysNO: S-nitrosohomocysteine

HPLC: High performance liquid chromatography

kDa: 10^3 dalton

mGST1: Microsomal glutathione S-transferase 1

NACysNO: S-nitroso-N-acetylcysteine

N-DEA: N-Nitrosodiethanolamine

NEM: N-Ethylmaleimide

$NO^{\bullet}$: Nitric oxide

$OONO^-$: Peroxynitrite

PAGE: Polyacrylamide gel electrophoresis

RSH: Thiol group

RSNOs: S-Nitrosothiols

SDS : Sodium dodecyl sulfate

1. Introduction

Nitrogen is an important compound of biomolecules and found in all living systems. A simple nitrogen species in the biological functions is nitric oxide. It is involved in a various of physiological functions including signalling and defence. High amounts of nitric oxide are toxic. Another toxic, respectively carcinogenic, class of nitrogen-containing compounds are the nitrosamines. They may be formed in endogenous reactions from secondary amines and nitrite under acidic conditions.

1.1. Nitric oxide synthesis and nomenclature.

Nitric oxide ($NO^{\bullet}$) is a short-lived simple inorganic diatomic free radical that plays an important role in the homeostatic regulation of the central nervous, immune and cardiovascular systems, and is synthesized by the constitutively expressed, calcium-dependent, cNO-synthase or endothelial NO-synthase (eNOS), upon stimulation by physiological mediators such as bradykinin (Moncada, *et al.* 1991; Nathan, 1992). These enzymes have been classified as either calcium and calmodulin-dependent (cNOS) or independent (iNOS)(Nathan, 1992). All three NOS (EC 1.14.13.39)(Bredt and Snyder, 1990; Nathan and Xie, 1994) isoforms have comparable specific activities of about 1µmol/min/mg protein at 37°C (Feldman, *et al*. 1993). Nathan and Xie, (1994) have advocated a simplified numerical nomenclature for NOSs.(Table 1).

Table 1. NOS nomenclature

Original designation	Functional designation*	Numerical designation
nNOS (constitutively expressed)	ncNOS	Type I
eNOS (constitutively expressed)	ecNOS	Type II
macNOS (inducible)	iNOS	Type III

* Preferred designation: original nomenclature derives from tissues from which the isoform was first purified and cloned: n = neuronal; e = endothelial and mac = macrophage. nNOS and eNOS are constitutively expressed in these cells while macNOS requires induction with cytokines. The functional designation incorporates the role of calcium in the regulation of NOS. nc refers to NOS, first identified in neurons (n) whose activity is calcium dependent (c); ec refers to NOS, first identified in endothelium (e) whose activity is calcium dependent (c); and i refers to NOS whose ativity is calcium independent. The numerical designation (I, II, III) is based on the order in which genes were cloned. (modified from Feelisch and Stamler,1996).

1.2. Nitric oxide; adducts and reactivity.

In mammalian cells, NOSs catalyze the sequential oxidation of the substrate L-arginine to the L-citrulline (figure 1). Many of the physiological actions of $NO^{\bullet}$ are mediated by cyclic guanosine monophosphate (cGMP)-dependent pathways through the activation of soluble guanylate cyclase (Moncada and Higgs, 1993; Denninger and Marletta, 1999). Secondary oxidants derived from $NO^{\bullet}$, leading to S-nitrosylation and nitration of endogenous biomolecules, may also be involved in various physiological and pathophysiological phenomena through cGMP-independent pathways (McAndrew, *et al.* 1997; Ischiropoulos,1998; Gaston, 1999).

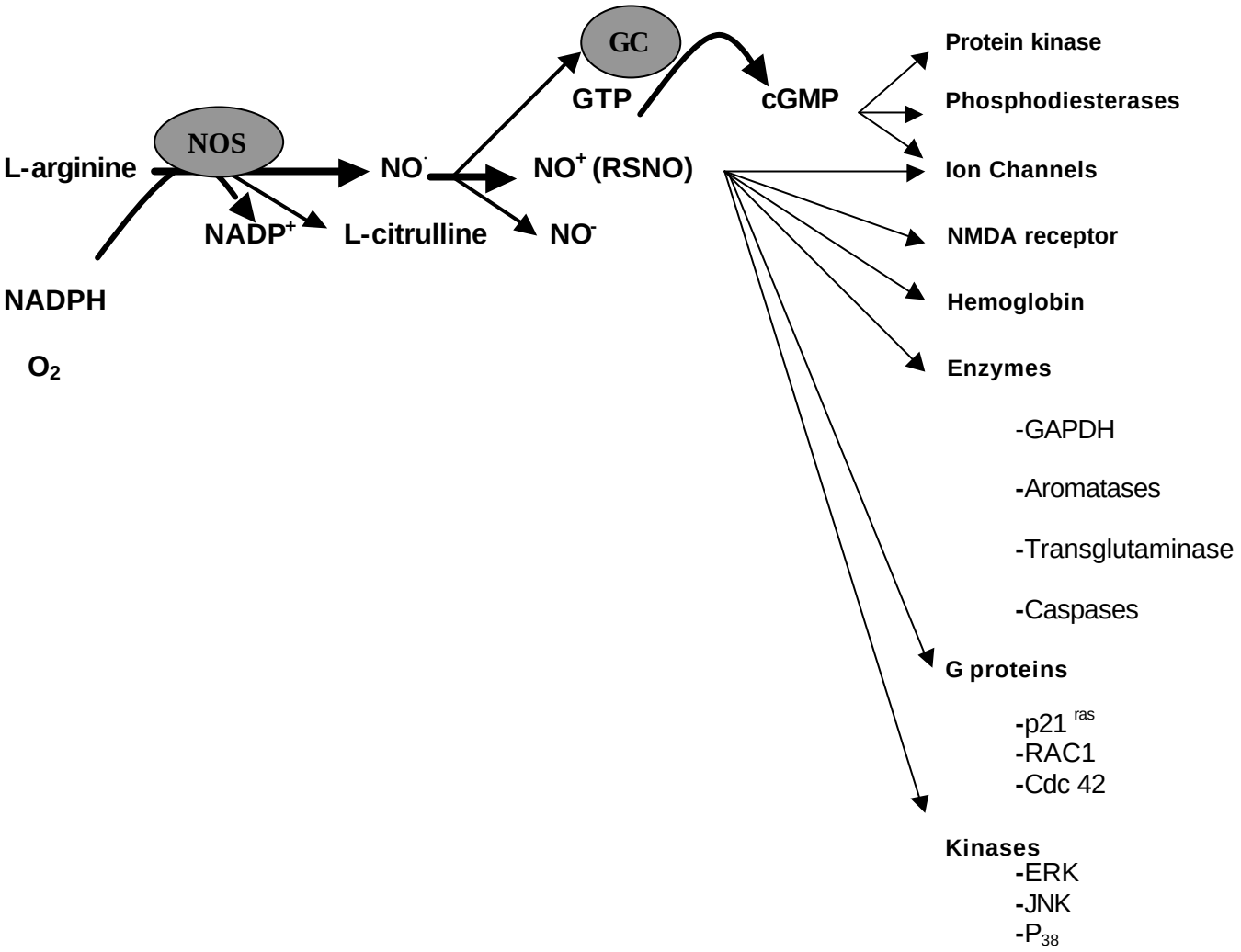


Figure 1. Biosynthesis of $NO^{\bullet}$ and nitrosothiols. NO-derived nitrosative compound can activate an array of target proteins either directly by S-nitrosylation or via the formation of intermediate nitrosothiols (RSNO).

The $NO^{\bullet}$ molecule, containing nitrogen in the +2 oxidation state, is in the sequence of the oxides ranging from -1 to +5; $NO_2^{\bullet}$ (common name nitrous oxide; IUPAC name dinitrogen monoxide); $NO^{\bullet}$ (nitrogen monoxide); N_2O_3 (dinitrogen trioxide); NO_2 and its dimer N_2O_4 (dinitrogen tetroxide); N_2O_5 (dinitrogen pentaoxide) and NO^- (nitroxyl anion).

The $NO^{\bullet}$ molecule reacts in biological systems with molecular oxygen, the superoxide radical anion, or transition metals (Stamler, 1992). The products of these reactions, higher nitrogen oxides (NO_x), peroxynitrite ($OONO^-$) and metal-nitrosyl adducts (Me-NO), have both various propensities for both toxicity and biological activity (Stamler, 1994). $NO^{\bullet}$ generation will lead to a variety of oxidation and reduction products with distinct life-times and biological activities. Metal- and thiol-containing regulatory proteins are preferential targets of NO^+ and also account for much of the $NO^{\bullet}$ associated bioactivity. It is now known that $NO^{\bullet}$ is not only responsible for endothelium dependent vascular relaxation but that it is also involved in central and peripheral neurotransmission and the defence mechanisms of the body as utilized by macrophages as well as many other roles (Battino and Clever, 1966; Wilhelm, *et al.* 1977; Schmidt and Walter, 1994; Nathan and Xie, 1994) .

The inflammatory process has been known as a risk factor for human cancer, particularly of the lung, bladder, colon, stomach, and female breast. It proposed that production of oxygen radicals, release of cytokines, and synthesis of prostaglandins and leukotrienes as biochemical modulators of the carcinogenic process. The discovery of $NO^{\bullet}$ as a product of cells in the immune system has implicated this compound in the mechanism of carcinogenesis, particularly when $NO^{\bullet}$ is overproduced over a long period of time. However, the chemical reaction of $NO^{\bullet}$

under physiological conditions and its key reactants toward biological important molecules relate to DNA damage and cytotoxicity (Ohshima and Bartsch, 1994).

$NO^{\bullet}$, a metabolic product of epithelial, endothelial, neuronal and phagocytic cells is produced under conditions associated with inflammation and $NO^{\bullet}$ might participate in carcinogenesis (Laskin, *et al.* 1994).

1.3. Toxic and mutagenic species generated by NO$^{\bullet}$

The fate of $NO^{\bullet}$ in biological system is governed by three processes: $NO^{\bullet}$ diffusion, transient autooxidation to $NO_2^{\bullet}$, and reaction with superoxide (Tannenbaum, 1994). Autooxidation of $NO^{\bullet}$ actually leads to formation of N_2O_3, a powerful electrophilic nitrosating agent (Lewis, 1995). The consequences of N_2O_3 formation are important because subsequent reactions lead to the formation of N-nitroso compounds, deamination and crosslinking of DNA (Lewis, 1995). N_2O_3 can also inactivate key enzymes involved in the protection against oxidative stress (Asahi, 1995) or in DNA repair (Wink and Laval, 1994).

Excess production of $NO^{\bullet}$, however, has been implicated as a cause of diverse pathophysiological conditions such as inflammation, neurodegenerative and cardiovascular dieseases, and cancer. These effects of $NO^{\bullet}$ have been also attributed to reactive nitrogen species such as NO_x and peroxynitrite ($ONOO^-$). Reactive nitrogen species can react with biomolecules such as proteins, DNA, and lipids through nitration and nitrosation, thus altering their functions (Ohshima, 1999; Ischiropoulos, 1998).

1.4. Peroxynitrite

Peroxynitrite, a strong oxidizing species, either by one-electron acceptance or as an oxene donor equivalent to a two-electron acceptor, is produced by the diffusion-limited reaction of nitric oxide and superoxide anion. Moreover, it decays to nitrate with a rate constant of 1.3 s^{-1} at 25^{o}C upon protonation to peroxynitrous acid (pK_a 6.7), which is a highly reactive compound yielding oxidizing and nitrating species (Beckman, 1990; Koppenol, 1992; Beckman, 1996; Kissner, 1997; Koppenol, *et al.* 1998; Sies, *et al.* 2000) (see reaction 1).

$$O_2^{\bullet} + NO^{\bullet} \longrightarrow ONOO^{-} \underset{}{\overset{H^+}{\rightleftharpoons}} HOONO \longrightarrow HO^{\bullet} + NO_2^{\bullet} \qquad (\mathbf{1})$$

NO imbalance in the endothelium may be used as a sign of vascular diseases such as atherosclerosis, hypertension and myocardial ischemia (Noack and Murphy, 1991; Sies, 1991). $NO^{\bullet}$ and derived species maintaining vascular tone (e.g., peroxynitrite) are cytotoxic to bacteria (Zhu, 1992). Macrophages and neutrophils produce a substantial amount of both nitric oxide and superoxide, which rapidly form peroxynitrite (Ischiropoulos, 1992). Moreover, such excessive production can damage normal tissue through the formation of 3-nitrotyrosine in proteins (Ohshima, 1990), an index of reactive nitrogen species, which is often increased under pathological condition such as inflammation and in different immunological process (Moncada, 1997; Nathan, 1997; Stuehr, 1997; Geller and Billiar,1998; Ischiropoulos, 1998; Marletta, 1998; Moncada, 1998).

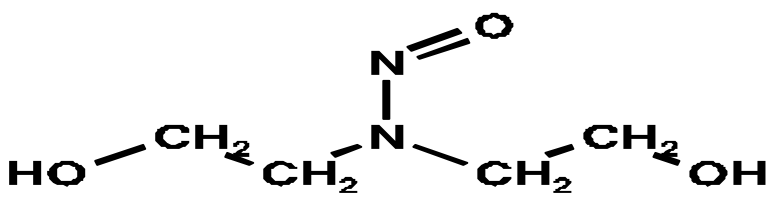
Since peroxynitrite can be formed in biological systems, its reactivity with cellular constituents has been investigated, and many destructive reactions with macromolecules such as DNA (Epe, 1996), lipids (Heinecke, 1997), or proteins (Ischiropoulos and Al-Mehdi, 1995) have been reported (for review see, Beckman

and Koppenol, 1996; Murphy, 1998). Typical reactions of proteins include thiol oxidation (Crow, 1995) or tyrosine nitrations (Crow, 1997) which have been observed under pathological conditions such as chronic rejection of transplanted organs (MacMillan-Crow, 1996) or neurodegenerative diseases (Beckman and Koppenol, 1996) and induce apoptosis through impairment of microtubule formation in cells (Eiserich, 1999).

1.5. N-Nitrosodiethanolamine

Carcinogenic nitrosamines have been reported to be formed by $NO^{\bullet}$, which is produced by inducible $NO^{\bullet}$ synthase expressed in cultured cells, including macrophages, hepatocytes, and neutrophils, upon stimulation by lipopolysaccharide and α-interferon (Iyengar, 1987; Miwa, 1987; Kosaka, 1989; Liu, 1992; Grisham, 1992 ; Ohshima, 1991). $NO^{\bullet}$ and reactive oxygen species produced in infected and /or inflamed tissues could contribute to the process of carcinogenesis by several mechanisms, including the formation of carcinogenic nitrosamines from $NO^{\bullet}$ (Ohshima and Bartsch, 1994; Liu and Hotchkiss, 1995; Tamir and Tannenbaum, 1996).

N-Nitrosodiethanolamine (see N-DEA structure) , is a potent liver carcinogen in experimental animals and is one of the most prevalent and abundant environmental occurring nitrosamines (Preussmann, 1982; Lijinsky, 1980).



(N-DEA)

It can be readily formed by the nitrosation of ubiquitous compounds such as di-or triethanolamine and their derivatives. N-DEA is a common trace contaminant in cosmetics, personal care items, tobacco products, metal working fluids, and pesticides (Fan, 1977a; Fan, 1977b; Chou and Havery, 1994).

Nitrosamines are known to require metabolic activation via the hydroxylation at the α-C-atom to exert their carcinogenic effect (Loeppky, 1999; Yang, 1990). The α-hydroxylation is mediated by cytochrome P450-dependent enzymes (Yang, 1990) and there is evidence that the carcinogenic activation of N-DEA also involves α-hydroxylation. But the first activation step involve the oxidation of the 2-hydroxyethyl group of N-DEA (**1**) to an aldehyde, N-(2-hydroxyethyl)-N-nitrosoethanal (NHEE, **2**), which is mediated by mammalian liver alcohol dehydrogenase (ADH) (see figure 2). NHEE preferentially occurs as the more stable hemiacetal N-nitroso-2-hydroxy-morpholine (NHMOR, **3**), further oxidation leads to the formation of N-(2-hydroxyethyl)-N-nitrosoglycine (NHEG, **4**)(Airoldi, *et al.*1983; Airoldi, 1984). The N-nitrosaminoaldehydes are highly reactive compounds possessing the ability of transferring their nitroso groups to other amines, namely, transnitrosation (Airoldi, *et al.*1984; Loeppky, *et al.* 1987) resulting in nitrosamine formation from secondary amines and diazonium ion formation (see figure 2). Chung and Hecht, (1985) showed that deoxyguanosine reacts with NHMOR to form an 1,N^2-glyoxal-deoxyguanosine adduct (**gG**). Moreover, NHMOR could deaminate the primary amino group in DNA, which would change base-pairing characteristics and cause crititical damage in DNA (Loeppky, 1987; 1993; 1999; Ngyuyen, *et al.*1992).

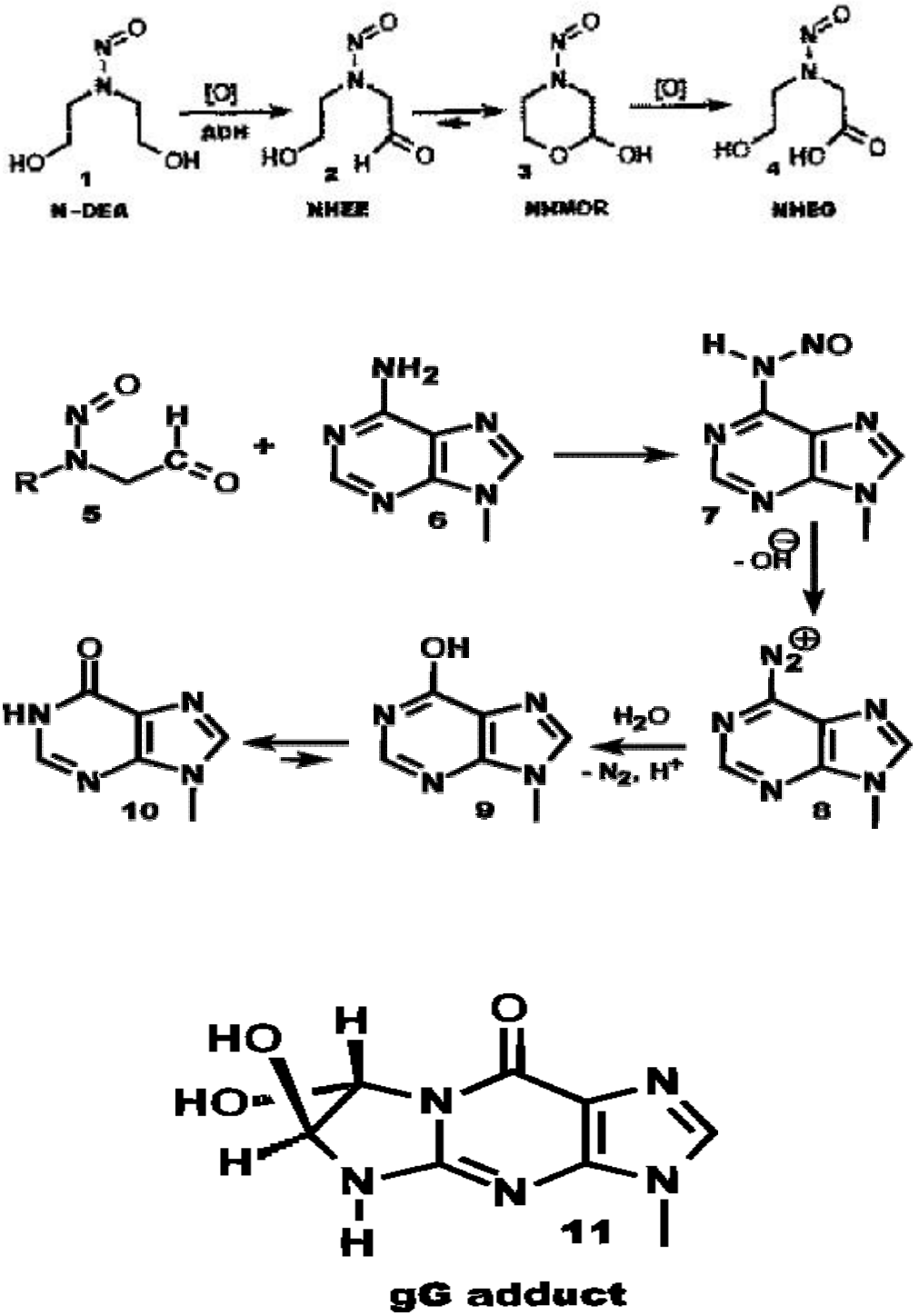


Figure 2. Nitrosamine activation scheme and the glyoxal-deoxyguanosine adduct formation.

1.6. S-Nitrosothiols

S-Nitrosothiols (RSNOs) were first synthesized in 1909 by Tasker and Jones. In 1974, Incze and co-workers showed that these compounds exert biological activity, e.g. antibacterial effects (Incze, 1974). The first studies in mammalian systems were performed in the early 1980s by Ignarro and co-workers, who demonstrated that RSNOs possess smooth muscle relaxant properties and inhibit of platelet aggregation (Ignarro, 1980). RSNOs were shown to be endogenous products in mammalian systems and identified in human and rabbit plasma (Stamler, 1992). RSNOs have also been found in human airway lining fluid (Gaston, 1993), and intracellularly in neutrophils (Abramson, 1993). It has become increasingly clear that RSNO derivatives of amino acids, peptides and proteins are natural products of the $NO^{\bullet}$ metabolism, e.g. S-nitrosoglutathione.

1.7. Properties and biochemical mechanism of action

The stability of RSNOs under physiological conditions varies as a function of the substituent of the thiol group (RSH) , pH, oxygen tension, redox state or the presence or absence of transition metals (Gaston, 1993; 1994; Mathews and Kerr, 1993; Radomeski, 1993; McAninly, 1993; Ignarro, 1981).

In view of their reactivity, RSNOs have been used as effective nitrosating agents in organic synthesis. Their nitrosating activity have important implications for biological systems, where nucleophils may increase RSNO decomposition through heterolytic mechanisms (Stamler, 1992). Such RSNO decomposition may predominate in many biological activities (Stamler, 1992; 1995).

S-Nitrosothiol-thiol exchange (i.e NO^+) transfer reactions, referred to as transnitrosation reactions (Feelisch, 1991), have been involved in the inhibition of glutamate-induced neurotoxicity (Lipton, 1993), the antimicrobial effects of various RSNOs compounds (Morris, 1984), and the inhibition of several sulfhydryl containing enzymes (Stamler,1995), and the mechanism of protein RSNOs activation *in vivo* (Stamler, 1997; Scharfstein, 1993).

1.8. Physiological RSNOs and their roles

The formation of RSNO has now been shown to occur in *in vivo* for an array of proteins (Butler, 1995; Lander, 1997). RSNOs like S-nitrosoalbumin, S-nitrosoglutathione (GSNO) and S-nitrosocysteine (CysNO) have been detected and quantified *in vivo* (Kluge, 1997) .

Moreover, GSNO and other RSNOs may inhibit enzymes associated with response to oxidative stress in eukaryotic cells, including glutathione peroxidase (Asahi, *et al.*1995; Fujii, *et al.* 1997), glutathione disulfide reductase (Becker, 1995) and γ–glutamyl cysteine synthase (Han, 1996).

1.9. Microsomal glutathione S-transferases

Glutathione S-transferases (GST, E.C. 2.5.1.18) comprise a group of phase II detoxication enzymes that occur abundantly in eukaryotic cells and also in prokaryotic organisms (Sies and Ketterer, 1988; Armstrong, 1997). These enzymes are a major defense systems against carcinogenic, mutagenic, toxic, and pharmacologically active electrophilic compounds (Chasseaud, 1979). These enzymes also mediate the protection against carcinogenic substances in our diet

(Hayes and Pulford, 1995). On the other hand, glutathione S-transferases have also been suggested to protect tumors from chemotherapy (Tew, 1994). Attesting to the importance of this enzyme system is the presence of many cytosolic isoenzymes in several related families (Mannervik and Widersten, 1995) as well as structurally and phylogenetically distinct membrane-bound glutathione S-transferases. Microsomal glutathione S-transferase 1 (mGST1) (Andersson, 1994) is a homotrimeric membrane protein (17.3 kDa) that also forms part of the so-called membrane-associated proteins in eicosanoid and glutathione metabolism superfamilly. This superfamily contains structurally and phylogenetically related enzymes involved in detoxication, protection from oxidative stress, and synthesis of prostaglandin E and cysteinyl leukotrienes (Jakobsson, 1999a,b).

Microsomal glutathione S-transferase 1 displays a unique ability to be activated (up to 15-fold) by sulfhydryl reagents such as N-ethylmaleimide (NEM), redox events, and proteolysis (Morgenstern, *et al.* 1979; 1987; 1989). The activated enzyme displays an increased catalytic efficiency at low glutathione concentration that occur during toxic insult (Andersson, 1995). Microsomal glutathione S-transferase 1 contains a single cysteine residue per subunit (DeJong, 1988; Morgenstern, 1985). This sulfhydryl, Cys49, resides on the cytoplasmic side of the endoplasmic reticulum and was demonstrated as the site of activation, since mutation to alanine prevented activation of the enzyme by NEM (Weinander, 1997).

1.10. Homocysteine

Homocysteine was discovered by duVigneaud (1952). Plasma total homocysteine, homocyst(e)ine, is the sum of the thiol containing amino acid, homocysteine and the homocysteinyl moiety of the disulfides homocystine and cysteine-homocysteine, whether free or bound to protein (Mudd and Lew, 1995). The concentration of homocyst(e)ine in blood, plasma, or serum is normally ≤ 16 µM (Malinow, 1989). In hyperhomocyst(e)inemia, the concentration of homocyst(e)ine is above normal: moderate, intermediate, and severe hyperhomocyst(e)inemias refer to plasma concentrations between 16 and 30, 31 and 100, and ≥ 100 µM, respectively (Kang, 1992).

An association between elevated plasma homocyst(e)ine concentration and atherosclerotic vascular disease has been observed in a number of epidemiological studies (McCully, 1969; Boushey, 1995). Hyperhomocyst(e)inemia may be caused by either genetic (defect of enzymes involved in the metabolism of methionine, mostly cystathionine ß-synthase or methylenetetrahydrofolate reductase) (Mudd and Levy, 1989; Kang, 1991) or dietary factors and occurs in up to 30% of patients with stroke, peripheral vascular disease or myocardial infarction (Boushey, 1995; Malinow, 1994). Subclinical deficiencies of folic acid, vitamin B-6 or B-12 elevate plasma homocyst(e)ine to levels associated with vascular disease (Selhub, 1993; Robinson, 1995; Selhub, 1996; Ubbink, 1996; Morrison, 1996). Supplementation of the diet with B vitamins is effective in lowering plasma homocyst(e)ine concentration (Ubbink, 1994).

Homocysteine can be viewed as an independent risk factor for atherosclerosis and is believed to exert its effects through a mechanism involving oxidative damage (Wall, 1980; Starkbaum and Harlan, 1986). Auto-oxidation of homocysteine in plasma

leading to the formation homocysteine-mixed disulfides, homocystine and oxygen-derived molecules, such as superoxide anion radical and hydrogen peroxide or hydroxyl radical are believed to account for the endothelial cytotoxicity (Mansoor, 1995). These reactive oxygen species generated during oxidation of homocysteine initiate lipid peroxidation (Heinecke,1987; Heinecke, 1988).

The antioxidant enzyme family, glutathione peroxidases, catalyse the reduction of both hydrogen peroxide and lipid hydroperoxides to their corresponding alcohols in a reaction mechanism that involves the oxidation glutathione (Flohe, 1988; Freedman, 1996). Homocysteine inhibits glutathione peroxidase activity *in vitro*. This effect occurs at concentrations of homocysteine that are pathophysiologically relevant (Upchurch, 1995).

The vasodilatory properties of the normal endothelial cell also altered by homocyst(e)ine, specifically with respect to endothelial nitric oxide (NO) production. Stamler and co-workers have shown that normal endothelial cells detoxify homocysteine by releasing NO or a related S-nitrosothiol which in turn leads to the formation of S-nitrosohomocysteine (Stamler, 1993). Endothelial cells respond to homocysteine by increasing their production of RSNOs, including S-nitrosohomocysteine which is formed under physiological conditions (Stamler, 1993). S-Nitrosation of homocysteine attenuates its pathogenicity by inhibiting sulfhydryl-dependent generation of H_2O_2. As other S-nitrosothiols, S-nitrosohomocysteine is a potent vasodilator, platelet agregation inhibitor and exhibits cytoprotective effects (Lipton, *et al.* 1993; Stamler, 1993).

1.11. Objective

Tissue injury in several human diseases is partly due to the formation of reactive oxygen and nitrogen species by activated cells. Recently, however, there has been considerable interest in the possible toxicity of nitrosothiols, the product of the reaction of $NO^{\bullet}$ and $ONOO^{-}$ and protein thiols and / or free thiols.

$NO^{\bullet}$ is a physiological mediator, produced in a wide variety of cells by NOSs (Stuehr, 1999), which is responsible for oxidative injury to host cells and tissues, in addition to its role in protecting the organism from external viruses and bacteria. $NO^{\bullet}$, the product of the NOSs and RSNO may react with O_2 and $O_2^{\bullet-}$ at relatively high rates with the subsequent formation of oxidants such as N_2O_3 and $ONOO^{-}$ (Patel, 1999). These oxidants bring about nitrosative stress (Hausladen, 1996), which is a form of oxidative stress associated with a change in the redox environment. Nitrosation of DNA bases, which results in oxidative deamination, is known to cause mutagenesis, as result of endogenous formation of N-nitroso compounds (Miwa, 1987; Lewis, 1995a) generated in cell culture (Lewis, 1995b), experimental animals (Leaf, 1991), and in humans (Leaf, 1990). S-Nitrosation of heme iron is involved in the inactivation of mitochondrial respiratory enzymes (Cleeter, 1994; Heales, 1999), leading to energy depletion. Nitration of tyrosine residue in Cu,Zn-superoxide dismutase (Ischiropoulos, 1992) lowers the ability of the enzyme to scavenge superoxide anion. It proposed that S-nitrosation of the ß-subunit of hemoglobin might influence the regulation of blood pressure (Jia, 1996; Stamler, 1997). It is suggested that these type of modifications, mediated by $NO^{\bullet}$, might be involved in the regulation of biological functions, which repesent the results of NO-induced oxidative injury.

Thiols are anti-oxidant molecules that play a major role in protecting cells from NO-induced oxidative injury (Padgett and Whorton, 1997; Clementi, 1998; Sies, 1999). GSH reacts with $NO^{\bullet}$ to form S-nitrosoglutathione in the presence of an electron

acceptor (Gow, 1997). GSH levels in cells exposed to $NO^{\bullet}$ decrease markedly (Clancy, 1994). GSNO is somewhat stable in pure aqueous solution in the dark (Mathews and Kerr,1993), but it is too unstable under biological conditions. This instability is due to that GSNO is degraded by Cu^{+} (Gorren, 1996; Singh, 1996a,b), superoxide (Aleryani, 1998; Trujillo, 1998), γ-Glutamyltranspeptidase (Kashiba, 1999) and the thioredoxin/thioredoxin reductase system (Nikitovic and Holmgren, 1996). It is known that glutathione S-transferases are inhibited by GSNO.

The aim of the present study was to examine :

(1) The formation of N-nitrosodiethanolamine *in vitro* upon the reaction of S-nitroso compounds and peroxynitrite with diethanolamine.

(2) The stability of S-nitrosothiols in the presence of thiol containing compounds in aqueous solutions and human serum.

(3) Isolation and characterisation of pig kidney microsomal glutathione S-transferase 1.

(4) The inhibitory effect of S-nitrosothiols and peroxynitrite towards the pig kidney microsomal glutathione S-transferase 1 and rat cytosolic glutathione S-transferases.

2. Materials and Methods

2. 1. Chemicals

Sodium nitrite, 1-chloro-2,4-dinitrobenzene (CDNB) and N-acetyl-L-cysteine were purchased from Merck (Darmstadt, Germany). DL-Homocysteine, L-cysteine, cysteinylglycine, glutathione, sulfanilamide, N-(1-naphthyl)-ethylenediamine, bathocuproine disulfonic acid, N-ethylmaleimide and glyceraldehyde-3-phosphate dehydrogenase were from Sigma (Deisenhofen, Germany). Monoclonal anti-nitrotyrosine antibodies were from Biomol (Hamburg Germany). Diethanolamine was from Aldrich-Sigma (Deisenhofen, Germany). Triton X-100 was from Fluka (Buchs, Switzerland). CM-Sepharose, Sephadex G-25, and G-100 were from Pharmacia (Uppsala, Sweden). Hydroxyapatite was from Bio-Rad laboratories (Richmond,CA). Immobilon (PVDF)-membrane was from (Millipore, Bedford, USA). Western blot alkaline phosphatase system was from Promega (Madison, WI)

2. 2. Methods

N-Nitrosodiethanolamine was synthesized from diethanolamine according to Preussmann, 1962. The compound was characterized by UV-spectroscopy and mass-spectrometry; purity was checked by HPLC.

2.2.1. Synthesis of peroxynitrite

Peroxynitrite was prepared by the reaction of acidified H_2O_2 with sodium nitrite using a quenched-flow reactor (Koppenol *et al.* 1996) and treated with manganese dioxide (1 mg/ml, 30 min at 4°C) to eliminate excess H_2O_2. Manganese dioxide was removed by centrifugation (for 10 min at 4°C and 15,000 g) and filtration (0.45 µm; Millipore). The final concentration was determined spectrophotometrically at 302 nm in 0.15 M NaOH ($\in_{302}$ = 1,670 M^{-1} cm^{-1}). The solution of peroxynitrite was stored at –80°C until use.

2.2.2. Synthesis of S-nitrosothiols

S-Nitrosocysteine, S-nitrosohomocysteine, S-nitrosocysteinylglycine, S-nitrosoglutathione, and S-nitroso-N-acetylcysteine were synthesized according to Mathews and Kerr, (1993). S-Nitrosothiols were prepared by combining 20 mM thiol in 0.1 N HCl and 20 mM sodium nitrite. After 5 min the mixture was neutralized with 1 M Tris (pH 7.0). The concentrations of the nitrosothiols were determined by measuring the absorbance at 330 nm (see Table 2 and Figure 3). Purity was checked by means of HPLC with UV-Vis detection (236 nm) and was more than 95%.

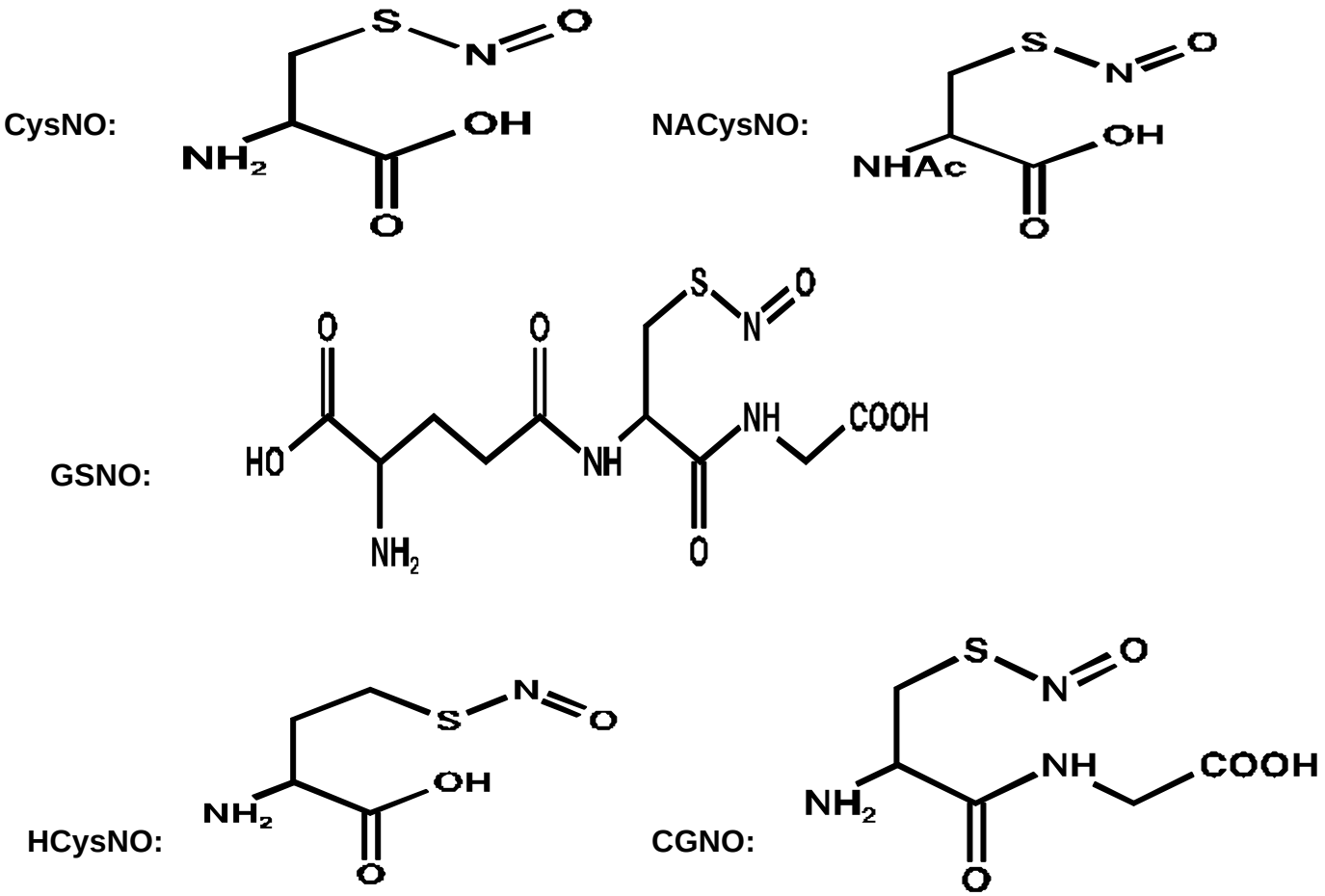


Figure 3. Structures of S-nitrosothiols. S-Nitrosocysteine (CysNO), S-nitroso-N-acetylcysteine (NACysNO), S-nitrosoglutathione(GSNO), S-nitrosohomocysteine (HCysNO), S-nitrosocysteinylglycine (CGNO).

Table 2 : Extinction coefficients of synthetic S-nitrosothiols at 330 nm.

Compound	Abbreviation	ϵ (M^{-1} cm^{-1})*
S-Nitrosocysteine	CysNO	594
S-Nitrosohomocysteine	HCysNO	915
S-Nitrosocysteinylglycine	CGNO	800
S-Nitrosoglutathione	GSNO	767
S-Nitroso-N-acetylcysteine	NACysNO	727

*: Mathews and Kerr, (1993).

2.2.3. Incubation of diethanolamine with S-nitrosothiols and peroxynitrite

The reactions of nitrosothiols or peroxynitrite with diethanolamine were carried out in 3.5 ml 10 mM phosphate buffer pH 7.0, in a glass tube under stirring at 37°C. The reaction mixtures contained 1.25 mM nitrosothiol (control), 1.25 mM nitrosothiol plus 1.25 mM diethanolamine, or 1.25 mM nitrosothiol plus 2.5 mM diethanolamine.
In an additional set of experiments S-nitrosohomocysteine (1.25 mM) was incubated with 1.25 mM and 2.5 mM diethanolamine in the presence of cysteine (1 mM) under the same conditions.

Peroxynitrite at final concentrations of 1 or 2 mM was incubated with 1 mM diethanolamine in 10 mM phosphate buffer pH 7.0 at 37°C. Stock solution of 100 mM peroxynitrite was used. The pH was 7.4 after addition of peroxynitrite.

2.2.4. Analysis of N-nitrosodiethanolamine and S-nitrosothiols

Aliquots (20 µl) of the reaction mixtures were taken at various time points and analyzed for N-nitrosodiethanolamine and S-nitrosothiol content by means of HPLC with a Merck-Hitachi HPLC system (pump 655A-12, UV-Vis-detector L-4200, Chromato-Integrator D2500; Merck, Darmstadt, Germany). For separation a reversed phase C-30 column 4.6 x 250 mm (YMC-carotenoid, YMC Ltd., Japan) was used

applying a flow rate of 1 ml/min; detection wavelength was 236 nm. N-Nitrosodiethanolamine and S-nitrosothiols were quantified according to the external standard method.

Various mobile phases were applied for the separation of N-nitrosodiethanolamine and S-nitrosothiols in different incubation mixtures. Separation of N-nitrosodiethanolamine and S-nitrosocysteine: 10 mM phosphate buffer pH 7.0; or S-nitrosocysteinylglycine: 10 mM phosphate buffer pH 2.5, 0.05% trifluoro-acetic acid, 6% methanol; or S-nitrosohomocysteine: 10 mM phosphate buffer pH 3.0, 0.1% trifluoro-acetic acid; or S-nitrosoglutathione: 10 mM phosphate buffer pH 2.5, 0.1% trifluoro-acetic acid, 6% methanol; or S-nitroso-N-acetylcysteine: 10 mM phosphate buffer pH 7.0, 6% methanol.

2. 2.5. Nitrite and nitrate analysis

Aliquots of the incubation mixtures were withdrawn at various time points and analyzed for nitrite and nitrate concentration according to Marzinzig *et al*. (1997).

2.2.6. Preparation and treatment of human serum with S-nitrosocysteine

Human serum was obtained from a healthy volunteer. Blood was collected by venipuncture. Serum was collected after centrifugation of the blood at 1,000 x g for 10 min. Serum was used immediately.

2.2.7. S-Nitrosothiol stability

S-Nitrosocysteine (10 µM) was added to 1 ml of human serum or 1 ml 10 mM phosphate buffer pH 7.0. Both mixtures contained 100 µM cysteine, and/or 1.4 mg/ml BSA and various concentrations of homocysteine (0, 10, 20 and 50 µM) and were incubated at 37°C. Aliquots of the incubation mixtures were withdrawn at various time

points and analyzed for S-nitrosothiols by HPLC. Samples were filtrated and 50 µl was injected onto a reversed phase C-30 column 4.6 x 250 mm (5 µm, YMC-carotenoid, YMC Ltd., Japan), the flow rate was 1 ml/min. The mobile phase was 10 mM phosphate buffer pH 2.5, 0.01 TFA; detection wavelength was 336 nm. S-Nitrosothiols were quantified according to the external standard method.

2.2.8. Transnitrosation between S-nitrosocysteine and homocysteine

All transnitrosation reactions were performed in 10 mM phosphate buffer pH 7.0 at 37°C . Either CysNO (100 µM) was mixed with homocysteine (100 µM) or HCysNO (100 µM) was mixed with cysteine (100 µM) for a various periods of times. At the indicated time points, aliquots were removed and immediately analysed by HPLC.

2.2.9. Preparation of microsomes from pig kidney.

Pig kidney was obtained from a local slaughter house and immediately placed in ice-cold 0.25 M sucrose containing 1 mM glutathione , 0.1 mM EDTA, 10 mM phosphate buffer, pH 7.0. The pig kidney cortex (100-200 g) was sliced into small pieces, and washed with 0.25 M sucrose,10 m M triethanolamine, pH 7.0 (buffer A), at 4°C, to obtain blood free tissue. The tissue was diluted 1 to 10 (w/v) in buffer A , minced and homogenized with a polytron tissue homogenizer and 10 times more homogenization with a teflon-glass homogenizer. The homogenate was centrifuged at 9,000 x g for 30 min at 4°C; the supernatant was further centrifuged at 105,000 x g for 40 min. The resultant supernatant was discarded and the remaining microsmal pellet was suspended in 0.1 M Tris-HCl buffer pH 7.2 and washed three times with the same buffer and centrifuged at 105,000 x g (40 min each). Then the microsomes were suspended in 0.25 M sucrose, 10 mM phosphate buffer pH 7.0, and stored at –80°C until use.

2.2.10. Glutathione S-transferase assays.

Glutathione S-transferase activity was measured according to Habig *et al.*1974, spectrophotometrically at 336 nm in a reaction system at 37°C, containing 1 mM glutathione or 5 mM N-acetyl-L-cysteine and 1 mM CDNB, 0.1 M phosphate buffer pH 7.0 and 0.1% Triton X-100. GSH conjugation activity of GST with CDNB and GSH or Nacys as substrate was determined. One unit of enzyme activity was defined as that conjugating 1 µmol CDNB/min at 37°C.

2.2.11. Microsomal glutathione S-transferase activation.

Activation of the microsomal glutathione S-transfrase by N-ethylmaleimide was performed as described (Morgenstern and DePierre,1982). Microsomes (1 mg/ml protein) were incubated with 2 mM NEM at room temperature for 5 min, the reaction was stopped by adding 2 mM GSH.

2.2.12. SDS-polyacrylamide gel electrophoresis.

SDS-polyacrylamide gel electrophoresis in discontinuous slab gels was performed according to the method of Laemmli (1970). The SDS concentration was 0.1% (w/v) and the spacer and the separating gels contained 3% and 15% (w/v) acrylamide, respectively. The protein samples were dissolved in 125 mM Tris-HCl buffer pH 6.8 containing 4% (w/v) SDS; 20% (w/v) glycerol; 10% (w/v) ß-mercaptoethanol and 0.02 % (w/v) bromophenol blue, mixed and boiled for 5 min. The samples and protein standards were loaded onto the gel. The gel was initially electrophoresed at room temperature with a constant voltage of 85 volts until the dye passed 3% stacking gel, then the voltage was raised to 150 volt.

2.2.13. Silver nitrate staining.

After electrophoresis the protein was visualised by silver staining according to Oakley, et al. (1980).The gel was fixed in 30% isopropanol and 10% acetic acid for 1 hr, then it was incubated in 30% isopropanol and 0.5 M sodium acetate for 1 hr, then the gel was washed three times (30 min each) with water. The gel was incubated in 0.1 % silver nitrate, 0.01 % formaldehyde for 1 hr and then it was washed shortly with water. The colour was developed with 2.5% sodium carbonate and 0.01% formaldehyde and reaction was stopped with 0.05% EDTA and then the gel was once washed with water.

2.2.14. Immunoblot analysis

Proteins were electrophoretically transferred from polyacrylamide gels onto nitrocellulose membrane (Multiphor Nova Pharmacia Transblot System) according to the method of Kyhse-Andersen (1984) or to PVDF-membrane according to Matsudaira (1987). The gel was equilibrated in 25 mM Tris; 0.2 M glycine; 20% methanol; pH 8.9 before electroplotting. Electroplotting was run for 1 hr at 60-100 mA (0.8 mA/cm^2 for 1-2 hr)(see figure 4).

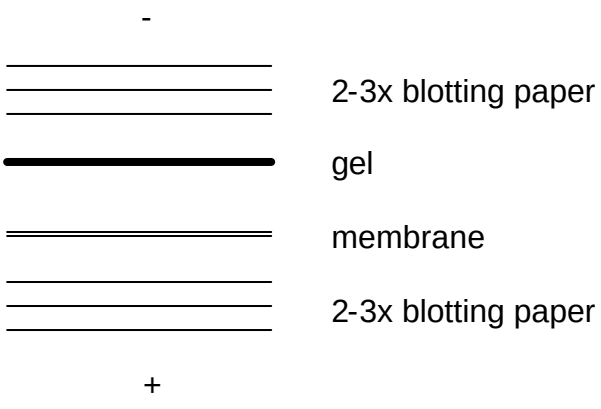


Figure 4. Scheme of the Western blotting technique.

After transfer, the nitrocellulose membrane was rinsed with TBST (TBS; 20 mM Tris, 137 mM NaCl, pH 7.6) containing 0.1% (v/v) Tween 20. Non-specific binding was blocked by placing the membrane in TBST supplemented with 5% (w/v) milk powder 1-3 hr at room temperature. The membrane was incubated with primary antiserum at optimum dilution 1:500 to 1:1000 in blocking buffer overnight at 4°C. The membrane was washed three times in TBST (10 min each) and then incubated for 1 hr at room temperature, with gentle shaking, in the same buffer containing alkaline phosphatase-conjugated antibody diluted 1:1000 to 1:5000. After treatment with alkaline phosphatase-conjugated antibody, the membrane was washed three times in TBST (5-10 min each) and twice in TBS, then immersed in development solution (10 ml TBS containing 66 µl nitrobluetetrazolium (50 mg/ml) and 33 µl 4-chloro-3-indolylphosphate (50 mg/ml). The blot was then washed once with distilled water, air-dried and photographed.

2.2.15. N-Terminal amino acid sequence.

The isolated pig kidney and liver microsomal glutathione S-transferase (50µg) was loaded onto SDS-gel electrophoresis and transfered to PVDF-membrane according to Matsudaira, (1987). PVDF-membrane was soaked in methanol and transfer buffer for 1 and 5 min, respectively.

2.2.15.1 Transfer-Buffer

10 mM 3-Cyclohexylamino-1-propanesulfonic acid (CAPS), pH 11.0 containing 10% (v/v) methanol to transfer the protein from the gel to membrane was used. For the transfer constant current (1 mA/cm^2 PVDF-membrane) was applied for 30 min (see 2.14).

2.2.15.2 PVDF-membrane staining:

PVDF-membrane was stained with coomasie blue R250, according to Batteiger, (1982).

Staining solution: 0.1% Coomassie blue R250; 25% methanol; 5% acetic acid.

Destaining solution: 25% methanol; 5% acetic acid.

The PVDF-membrane was soaked for 5 min in staining solution and then destained several times with destaining solution. The membrane was dried with a filter paper and stored at –20°C until use.

2.2.15.3 Amino acid sequence

The protein band was cut from the stained PVDF and subjected to automated Edman degradation using an Applied Biosystems model pulsed liquid sequencer, model 473A.

2.2.16. Protein determination.

2.2.16.1. According to Lowry

Protein concentration in microsomes was determined according to Lowry *et al.* (1951).

Solution A: 2% Na_2CO_3 . 10 H_2O in 0.1 NaOH.

Solution B: 0.5% Cu SO_4 . 5 H_2O and 1% Na_3-citrate.

Solution C: 25 ml “A” and 0.5 ml “B” (prepared always fresh).

BSA: 0.1 mg/ml (stock solution)

Folin- Phenol reagent 1:1 in H_2O diluted.

The BSA – standards contain 25, 50, 75 and 100 µl BSA stock solution and 100 µl H_2O as a blank. The samples and standards were completed to 100 µl final volume with H_2O and mixed with 1 ml solution “C”. After 10 min, 100 µl Folin reagent was

added and mixed immediately and incubate for 30 min at room temperature and then measured at 750 nm against a blank.

2.2.16.2. According to Bensadoun and Weinstein.

The protein treated with Triton X-100 was measured according to Bensadoun and Weinstein, (1976).

Solution A: 2% Na_2CO_3 . 10 H_2O in 0.1 NaOH.

Solution B: 0.5% Cu SO_4 . 5 H_2O and 1% Na-citrate.

Solution C: 25 ml "A" and 1 ml "B" (prepared always fresh).

BSA-Standard: 0.1 mg/ml (stock solution).

10% TCA

2% Na-deoxycholate.

Folin-Phenol reagent 1:1 in H_2O diluted.

The BSA – standards contain 50, 100, 150 and 200 µl BSA stock solution and 750 µl H_2O as a blank. The samples and standard were completed to 750 µl final volume with H_2O. 7 µl Na-deoxycholate was added, mixed and incubated for 15 min at room temperature, 0.6 ml TCA was added and incubated for 30 min at 4°C and then centrifuged at 10,000 xg for 5 min . One ml solution "C" was added to the pellets and vortex-mixed immediately. After 10 min, 100 µl Folin reagent was added and mixed immediately and incubate for 30 min at room temperature and then measured at 750 nm against a blank.

2.2.17. Purification of microsomal glutathione S-transferase.

Pig kidney microsomal glutathione S-transferase was purified by the method of Mogenstern, *et al.* (1982). The microsomes (100-200 mg) were solubilized by adding dropwise 15% Triton X-100 solution, followed by stirring for an additional 30 min on

ice. The solubilized microsomal sample was applied to a hydroxyapatite column (1.6 x 10 cm) equilibrated with 0.01 M potassium phosphate buffer pH 7.0, containing 0.1 mM EDTA, 20% glycerol, 1% Triton X-100, and 1 mM GSH (buffer A). The sample was eluted with a linear gradient of 0.01-0.3 M phosphate buffer pH 7.0 , 0.1 mM EDTA, 20% glycerol, 1% Triton X-100, and 1 mM GSH.

Two fractions exhibiting glutathione S-transferase activity were obtained on hydroxyapatite column chromatography and were further purified as follows: after exchange of the buffer through a sephadex G-25 column (2.6 x 60 cm), fraction I was chromatographed on S-hexylglutathione affinity chromatography column and eluted with 0.2 M NaCl, 20 mM GSH in 50 mM Tris buffer pH 9.6. The fractions exhibiting glutathione S-transferase activity were frozen at –80°C until use. Fraction II was applied to a CM-Sepharose column (1.6 x 13 cm), after desalting on a sephadex G-25 column and eluted with a linear gradient of 0.0-0.2 M KCl in buffer A. The fractions exhibiting glutathione S-transferase activity were frozen at –80°C until use.

2.2.18. Influence of S-nitrosothiols and peroxynitrite on glutathione S-transferase activity

In order to study the influence of S-nitrosothiols (i.e. CysNO, HCysNO) and peroxynitrite on glutathione S-transferase activity, the following procedure was used. Glutathione S-transferase (2 Units) was pre-incubated for 10 min at 25°C with different concentration of S-nitrosothiols and peroxynitrite. At the end of exposure, aliquots of the pre-incubated protein were taken and glutathione S-transferase activity was determined. Control was run without S-nitrosothiols and peroxynitrite.

3. Results

3.1. HPLC analysis of S-nitrosothiols and N-nitrosodiethanolamine

In separate experiments five different S-nitrosothiols, including S-nitrosocysteine, S-nitrosoglutathione, S-nitrosohomocysteine, S-nitrosocysteinylglycine and S-nitroso-N-acetylcysteine or peroxynitrite were incubated with diethanolamine. The decomposition of S-nitroso compounds and the formation of N-nitrosodiethanolamine generated *via* sulfur-to-nitrogen transnitrosation was followed by HPLC. Typical chromatograms obtained from an incubation mixture of S-nitrosocysteine and diethanolamine at different time points are shown in figure 5A. Figure 5B shows typical chromatograms obtained from incubation mixtures containing either S-nitrosocysteinylglycine, S-nitrosohomocysteine, S-nitrosoglutathione or S-nitroso-N-acetylcysteine together with diethanolamine. The retention times are assigned in the chromatograms and are identical to those observed with reference compounds. The compounds of interest are baseline-separated and are assigned in the chromatograms. The signal at a retention time of 2.8 min originates from the sample solvent front and from cystine which was formed during incubation (figure 5A); no free thiols were detectable in the mixtures using Ellman´s reagent. The formation of disulfides upon decomposition of S-nitrosothiols has been described in the literature as a major reaction pathway (Barnett et al, 1994).

N-Nitrosodiethanolamine was identified by comparison of retention times with the synthetic reference compound and by UV spectroscopy. The peak assigned to N-nitrosodiethanolamine was isolated and further characterized by mass spectrometry. The mass spectrum obtained from the isolated fraction was identical with the spectrum of a synthetic reference compound (MH^+ at m/z 135) and exhibited a similar fragmentation pattern. Characteristic fragments were observed at m/z 117

(parent ion - H_2O), m/z 104 (parent ion – NO) and m/z 74 (parent ion – NO – OH-CH_2).

3. 2. Transnitrosation from S-nitrosocysteine to diethanolamine

The transnitrosation from S-nitrosothiols to diethanolamine yielding N-nitrosodiethanolamine was investigated at pH 7.0 in 10 mM phosphate buffer at 37°C. The concentration of the added S-nitroso compounds was 1.25 mM in all experiments; diethanolamine was added at 1.25 mM or at 2.5 mM.

Without any diethanolamine present in the mixture (control), S-nitrosocysteine decomposed spontaneously with a half-life of about 35 min (figure 6A); after 60 min of incubation the concentration of the parent compound had decreased to 0.36 mM. When diethanolamine (1.25 mM) was present (figure 6A), S-nitrosocysteine decomposed more rapidly; the half-life then was 25 min, and only 0.21 mM was left at 60 min (Table 3). In the presence of diethanolamine (1.25 mM), N-nitrosodiethanolamine was formed to yield 51 µM after 80 min, a transnitrosation efficacy of about 4%. Nitrite levels also increased during incubation and accounted for about 45% of NO-equivalents of S-nitrosocysteine after complete decomposition; no nitrate was detected. When the concentration of diethanolamine was increased to 2.5 mM, the half-life of S-nitrosocysteine was further shortened (figure 6B). After 30 min the compound was fully consumed, and more than 140 µM of N-nitrosodiethanolamine had formed (transnitrosation efficacy of 11%).

3. 3. Transnitrosation from S-nitrosocysteinylglycine to diethanolamine

Very similar results were obtained when S-nitrosocysteinylglycine was used as a transnitrosating agent (figure 7). The half-life of the compound without diethanolamine (control) was also about 30 min; upon addition of diethanolamine

(1.25 mM) the half-life was shortened to about 15 min. After 50 min of incubation S-nitrosocysteinylglycine was practically fully consumed, and 38 µM N-nitrosodiethanolamine had formed (transnitrosation efficacy 3%) (figure 7); nitrite accounted for 43% of the NO equivalents in the mixture. The decomposition of S-nitrosocysteinylglycine was accelerated in the presence of 2.5 mM diethanolamine (Table 3). The yield of N-nitrosodiethanolamine was 90 µM after 60 min (transnitrosation efficacy 8%) (Table 3).

Other S-nitrosothiols investigated were more stable under the same conditions (Table 3). Neither in the presence nor absence of diethanolamine a statistically significant decomposition of S-nitrosohomocysteine, S-nitrosoglutathione and S-nitroso-N-acetylcysteine was observed within 210 min of incubation. Differences between the levels of nitrosothiols at t = 0 min and t = 210 min are within the coefficient of variation of the analytical method, which is 4%. It took several days until the compounds were decomposed (see figure 5b). Less than 4.4 µM N-nitrosodiethanolamine was found after 210 min which corresponds to a transnitrosation efficacy of less than 0.4%; only small amounts of nitrite where detected.

3.4. Transnitrosation from S-nitrosohomocysteine to diethanolamine in the presence of cysteine

S-Nitrosohomocysteine was incubated with 1.25 mM diethanolamine in the presence of 1 mM L-cysteine, and the loss of S-nitrosohomocysteine as well as the formation of N-nitrosodiethanolamine was followed (figure 8A). When L-cysteine was present in the mixture, the level of S-nitrosohomocysteine decreased rapidly with a half-life of 45 min; after 150 min all S-nitrosohomocysteine was consumed. The level of N-nitrosodiethanolamine in the mixture increased up to 30 µM at 150 min

(transnitrosation efficacy of 2.4%). S-Nitrosocysteine was formed as an intermediate in the reaction, reaching maximum concentration of 0.55 mM at 30 min (figure 8a). In the presence of L-cysteine and 2.5 mM diethanolamine, S-nitrosohomocysteine decomposed with a similar time course, and the intermediate formation of S-nitrosocysteine was also comparable between both experiments; however; more N-nitrosodiethanolamine (59 µM) was generated (transnitrosation efficacy of 4.7%)(figure 8B).

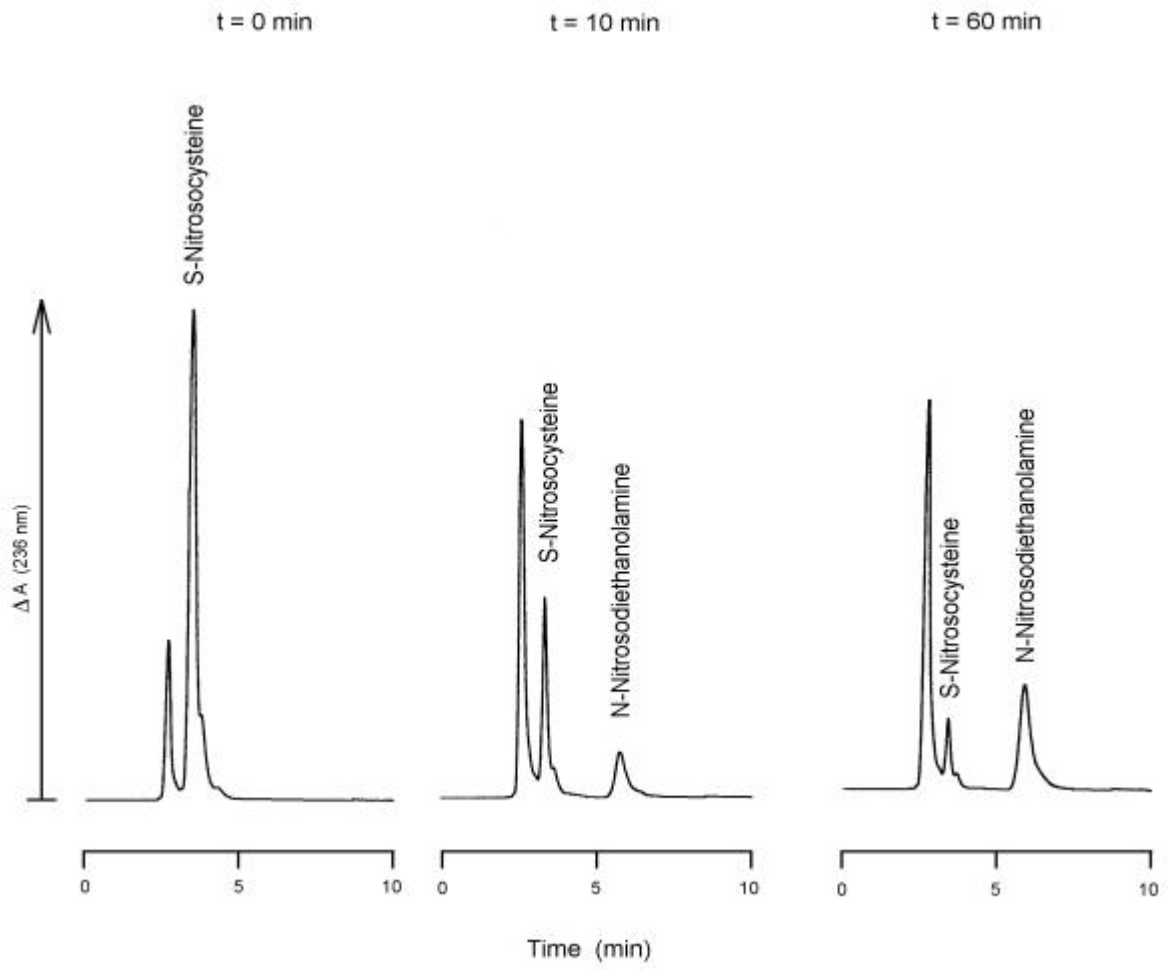


Figure 5A. HPLC traces obtained from an incubation mixture of S-nitrosocysteine and diethanolamine. S-nitrosocysteine and diethanolamine were1.25 mM in 10 mM phosphate buffer pH 7.0 at 37°C. Samples were taken at 0, 10 and 60 min.Separation was achieved with a reversed phase C-30 column 4.6 x 250 mm (YMC-carotenoid, YMC Ltd., Japan); flow rate of 1 ml/min, detection wavelength 236 nm. 10 mM phosphate buffer pH 7.0 was used as mobile phase.

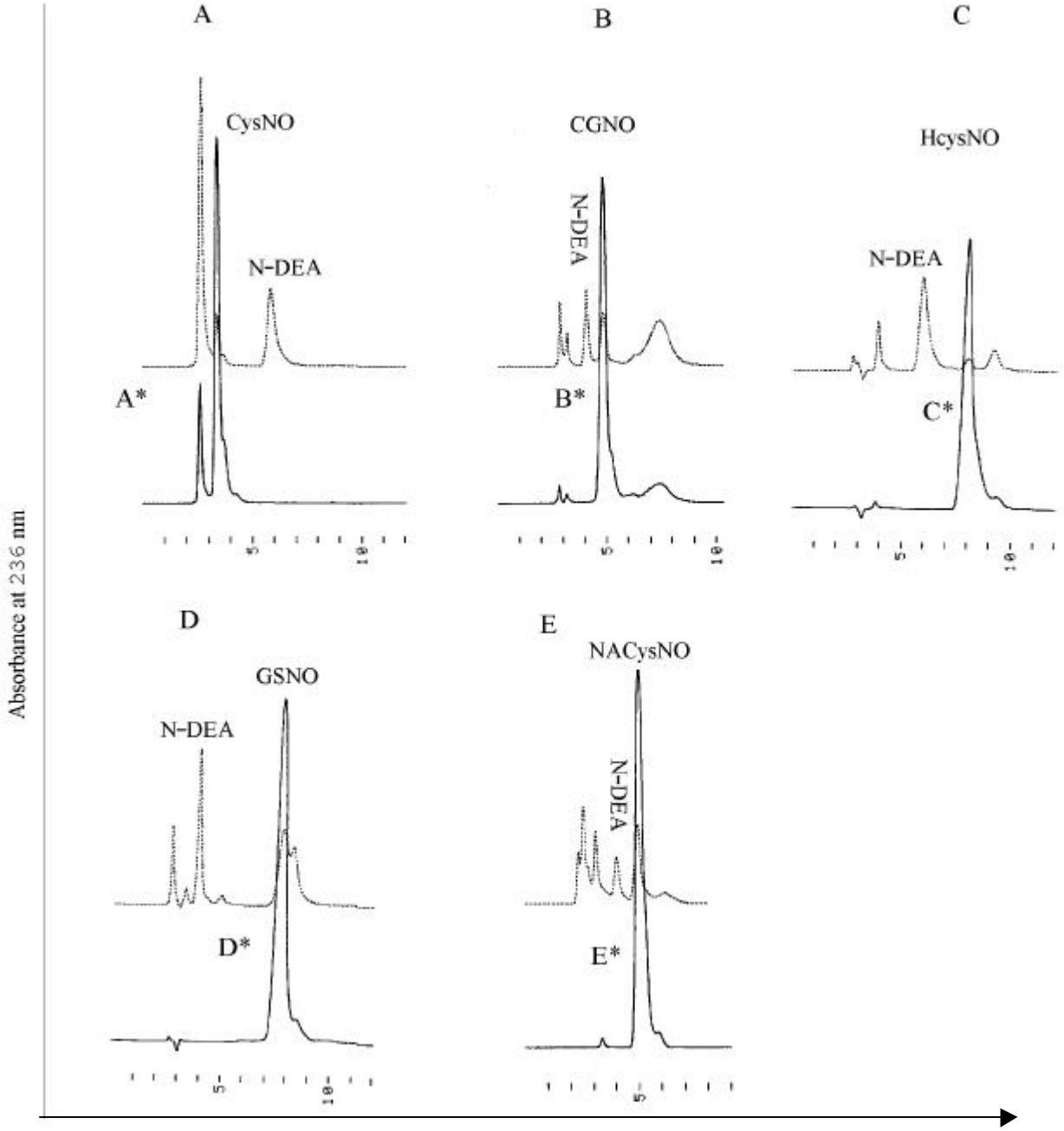
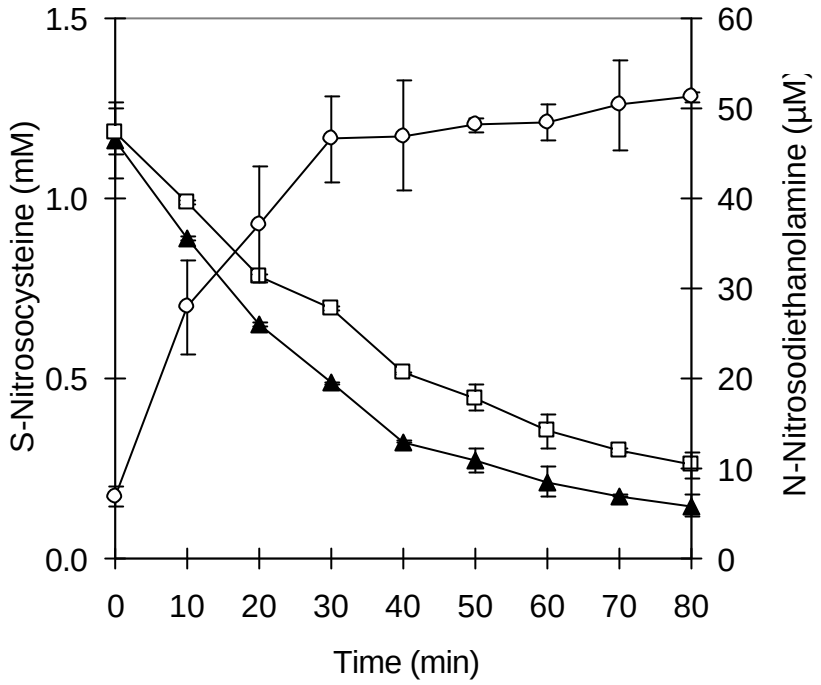


Figure 5B. HPLC chromatograms obtained from the reaction of S-nitrosothiols and diethanolamine in 10 mM phosphate buffer pH 7.0 at 37°C. Trace A* and A : CysNO / DEA at 0 and 60 min; trace B* and B : CGNO / DEA at 0 and 50 min; trace C* and C : HCysNO / DEA at 0 and 124 hr; trace D* and D : GSNO / DEA at 0 and 148 hr; trace E* and E : NACysNO / DEA at 0 and 172 hr. Separation was achieved as described in materials and methods.

(A)



(B)

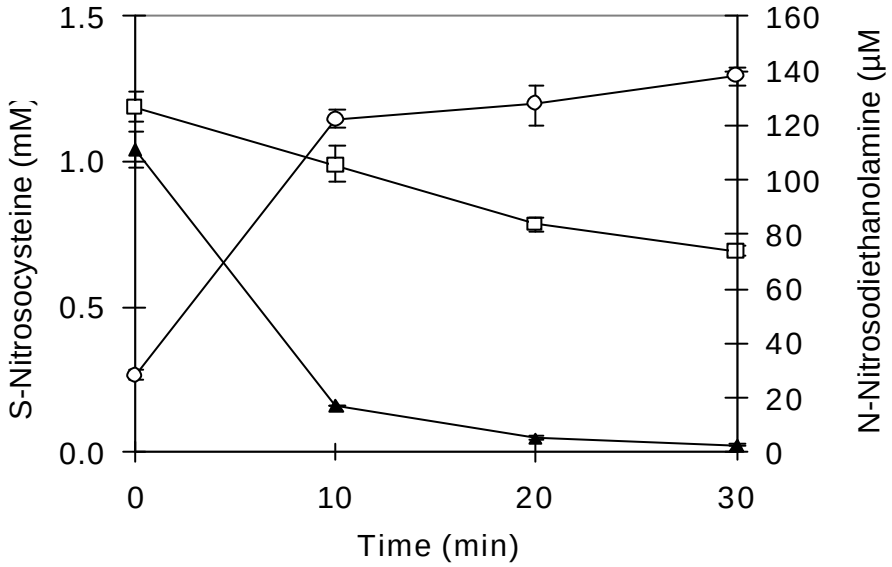
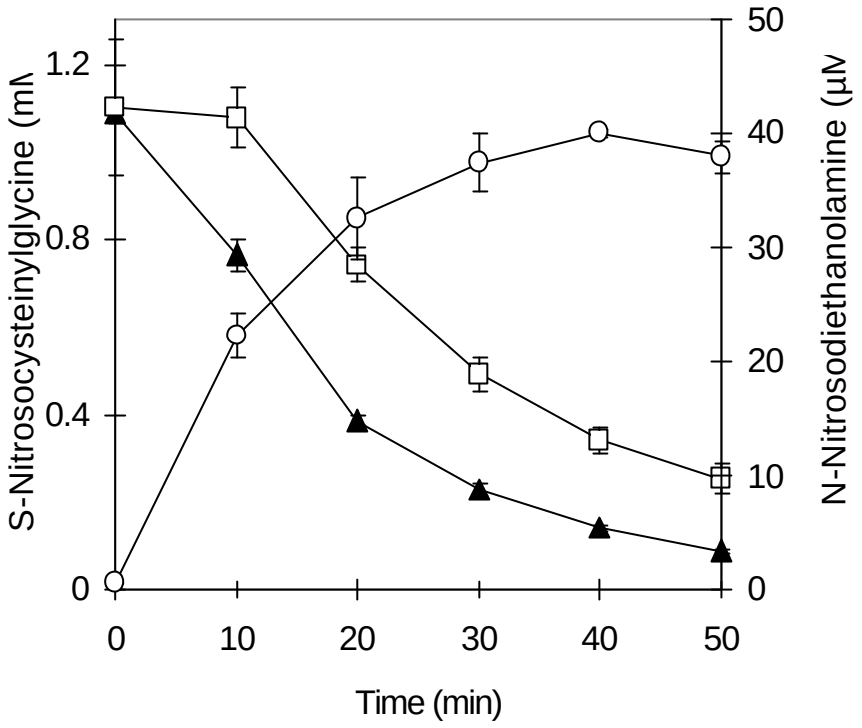


Figure 6. Formation of N-nitrosodiethanolamine and loss of S-nitrosocysteine. (A) Reaction mixture containing 1.25 mM S-nitrosocysteine in 10 mM phosphate buffer pH 7.0 at 37°C; S-nitrosocysteine and N-nitrosodiethanolamine were measured at different time points as described in experimental procedure. S-nitrosocysteine (control) ( ), S-nitrosocysteine with 1.25 mM diethanolamine (?). N-nitrosodiethanolamine (❍) formed from S-nitrosocysteine and 1.25 mM diethanolamine. B: As in A; but concentration of diethanolamine was 2.5 mM. Results are given as means of three independent experiments ± SD.

(A)



(B)

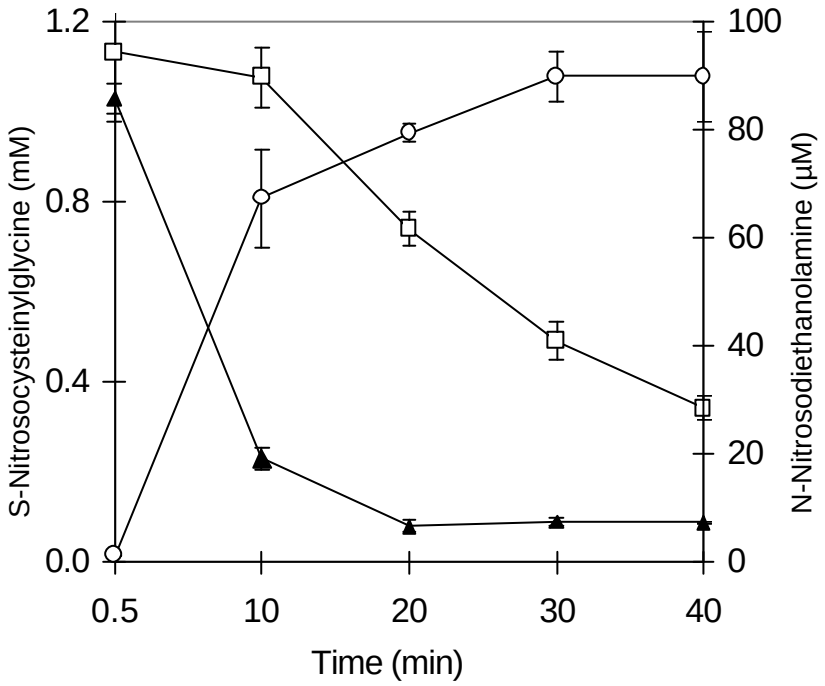
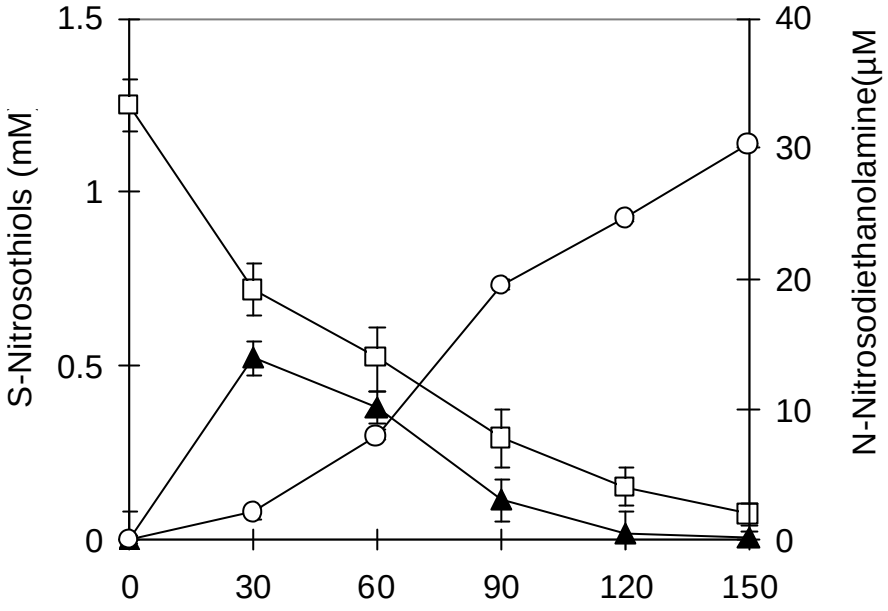


Figure 7. Formation of N-nitrosodiethanolamine and loss of S-nitrosocysteinylglycine. A: Reaction mixture containing 1.25 mM S-nitrosocysteinylglycine in 10 mM phosphate buffer pH 7.0 at 37°C; S-nitrosocysteinylglycine and N-nitrosodiethanolamine were measured at different time points as described in experimental procedure. S-nitrosocysteinylglycine (control) ( ), S-nitrosocysteinylglycine with 1.25 mM diethanolamine (**?**) and N-nitrosodiethanolamine (❍) formed from 1.25 mM diethanolamine. B: As in A; but concentration of diethanolamine was 2.5 mM. Results are given as means of three independent experiments ± SD.

(A)



(B)

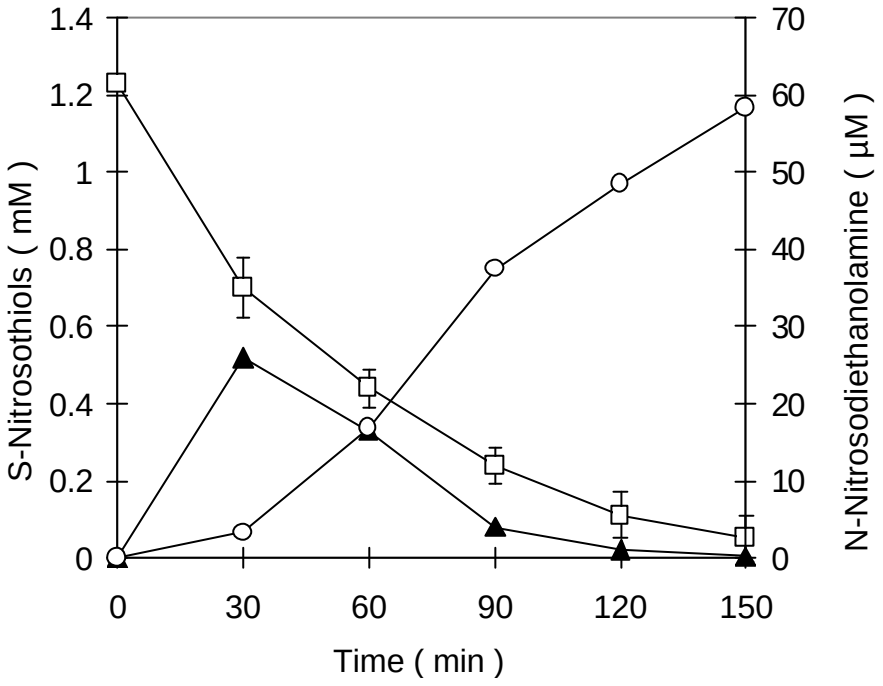


Figure 8. Formation of N-nitrosodiethanolamine and loss of S-nitrosohomocysteine in the presence of cysteine. A: Reaction mixture containing 1.25 mM S-nitrosohomocysteine and 1 mM cysteine in 10 mM phosphate buffer pH 7.0 at 37^{O}C; S-nitrosohomocysteine, S-nitrosocysteine and N-nitrosodiethanolamine were measured at different time points as described in experimenta procedure. S-nitrosohomocysteine ( ), S-nitrosocysteine (?), N-nitrosodiethanolamine (❍). B: As in A; but concentration of diethanolamine was 2.5 mM. Results are given as means of three independent experiments ± SD.

3.5. Nitrite formation during the reaction of S-nitrosothiols with diethanolamine

3.5.1. Nitrite formation from the reaction of S-nitrosocysteine with diethanolamine

During the reaction of diethanolamine with S-nitrosothiols nitrite is formed. Incubation of S-nitrosocysteine alone in phosphate buffer leads to spontaneous decomposition, and a time-dependent formation of nitrite. Nitrite level was increased from 36.2 (t=0 min) to 361 µM after 80 min (figure 9, table 3). Addition of 1.25 mM diethanolamine to S-nitrosocysteine resulted in an increased formation of nitrite reaching 550 µM after 80 min of incubation. When the diethanolamine concentration raised to 2.5 mM, the nitrite level reached 525 µM after 40 min of incubation. The major NO reaction product was NO_2^- and accounted for about 45% of NO equivalents derived from S-nitrosocysteine (figure 9).

3.5.2. Nitrite formation from the reaction of S-nitrosocysteinylglycine with diethanolamine

Very similar results were obtained when S-nitrosocysteinylglycine was incubated with diethanolamine. Without diethanolamine, nitrite level reached about 398 µM after 110 min. Incubation of S-nitrosocysteinylglycine with 1.25 mM diethanolamine lead to 634 µM nitrite after 110 min. When 2.5 mM diethanolamine was added to reaction mixture, nitrite level reached 615 µM after 50 min. Nitrite was the major NO product and accounted for about 49% of NO equivalents derived from S-nitrosocysteinylglycine (figure 10).

3.5.3. Nitrite formation from the incubation of S-nitrosohomocysteine, S-nitrosoglutathione, and S-nitroso-N-acetylcysteine with diethanolamine

As shown in figure 11 and table 3 incubation of S-nitrosohomocysteine without or with diethanolamine (1.25, 2.5 mM) resulted in a time dependent formation of nitrite, reaching 18.8, 45.8 and 79.9 µM after 210 min and accounted for 1.5, 3.7 and 6.4% of the NO equivalents in the reaction mixture, respectively.

The increases in nitrite levels are low as compared to the reactions of S-nitrosocysteine and S-nitrosocysteinylglycine, respectively. The stability of S-nitrosohomocysteine. Comparably low nitrite levels were found for S-nitrosoglutathione and S-nitroso-N-acetylcysteine which are also relatively stable under the same conditions (see table 3).

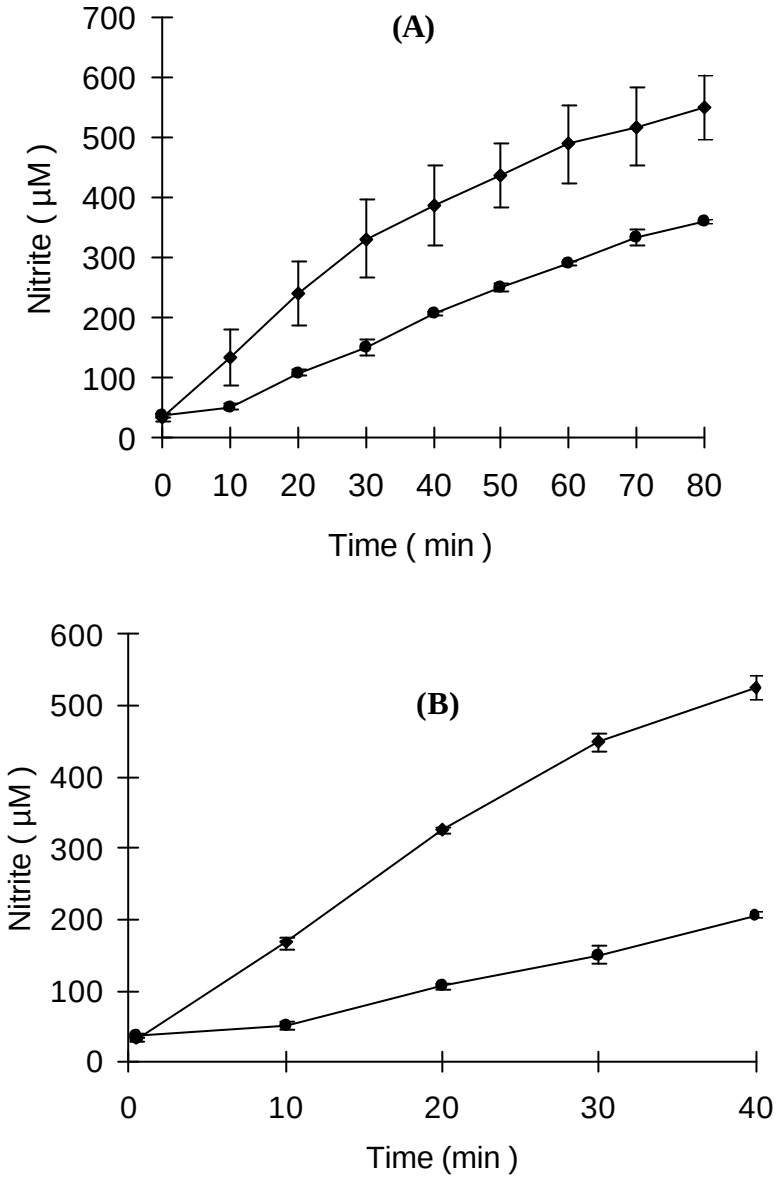


Figure 9A: Time course of the formation of nitrite during the reaction of 1.25 mM S-nitrosocysteine (CysNO) and 1.25 mM diethanolamine (DEA) in 10 mM phosphate buffer pH 7.0, at 37°C. B : Nitrite formation during the reaction of 1.25 mM CysNO and 2.5 mM DEA. Results are given as mean of three experiments ± SD. Nitrite formation with DEA (◆), nitrite formation without DEA (●).

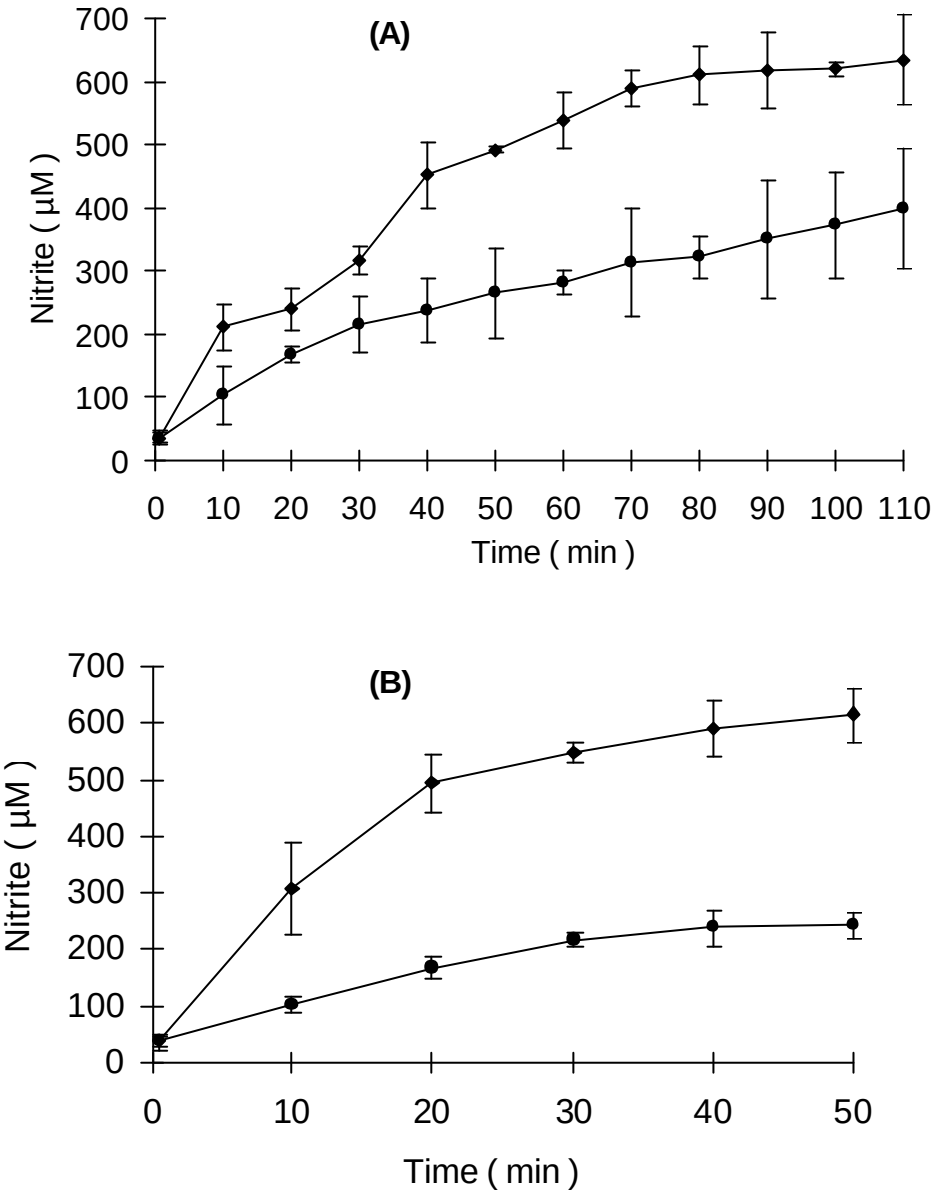


Figure 10A. Time course of the formation of nitrite during the reaction of 1.25 mM S-nitrosocysteinylglycine (CGNO) and 1.25 mM diethanolamine (DEA) in 10 mM phosphate buffer pH 7.0, at 37°C. B: Nitrite formation during the reaction of 1.25 mM CGNO and 2.5 mM DEA. Results are given as means of three experiments ± SD. Nitrite formation with DEA (◆), nitrite formation without DEA(●).

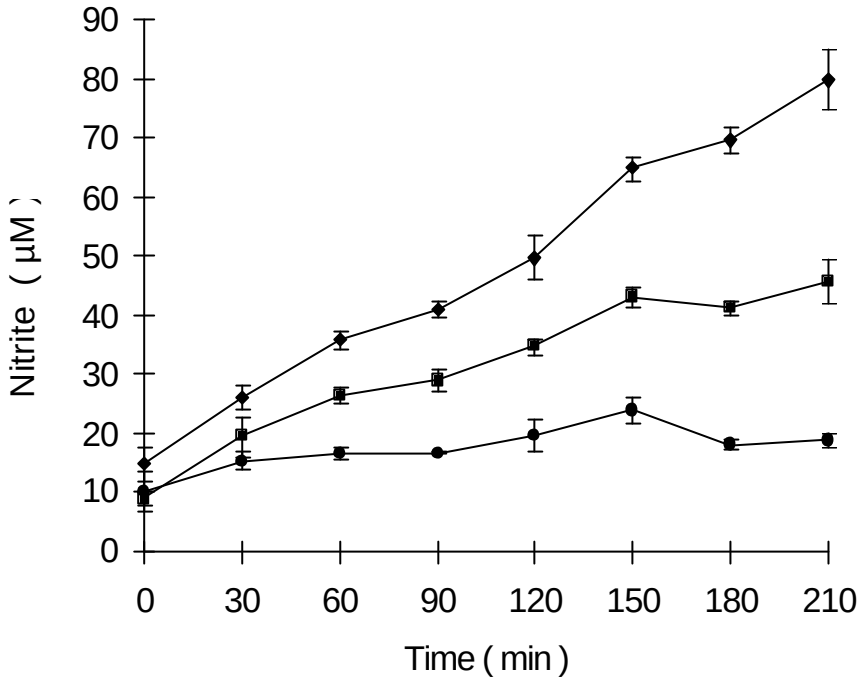


Figure 11. Time course of the formation of nitrite during the reaction of S-nitrosohomocysteine (HCysNO) and diethanolamine (DEA). Reaction mixtures contained 1.25 mM HCysNO and 1.25 mM DEA in 10 mM phosphate buffer, pH 7.0 at 37° C (■), 1.25 mM HCysNO and 2.5 mM DEA (◆) and 1.25 mM HCysNO (control) (●). Results are given as means of three experiments ± SD.

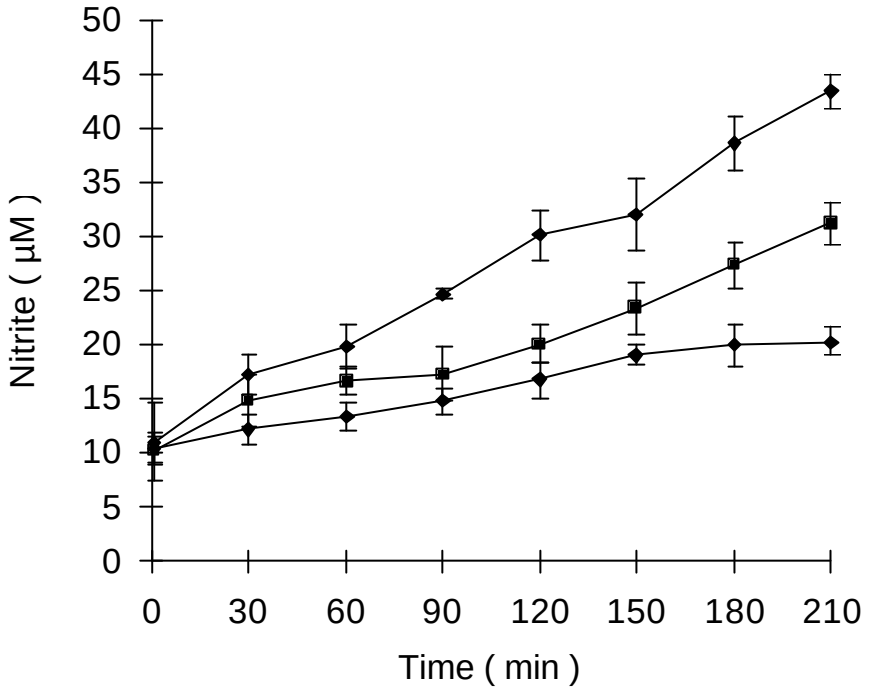


Figure 12. Time course of the formation of nitrite from the reaction of S-nitrosoglutathione (GSNO) and diethanolamine (DEA). Reaction mixtures contained 1.25 mM GSNO and 1.25 mM DEA in 10 mM phosphate buffer, pH 7.0 at 37° C (■), 1.25 mM GSNO and 2.5 mM DEA (◆) and 1.25 mM GSNO (control) (●). Results are given as means of three experiments ± SD.

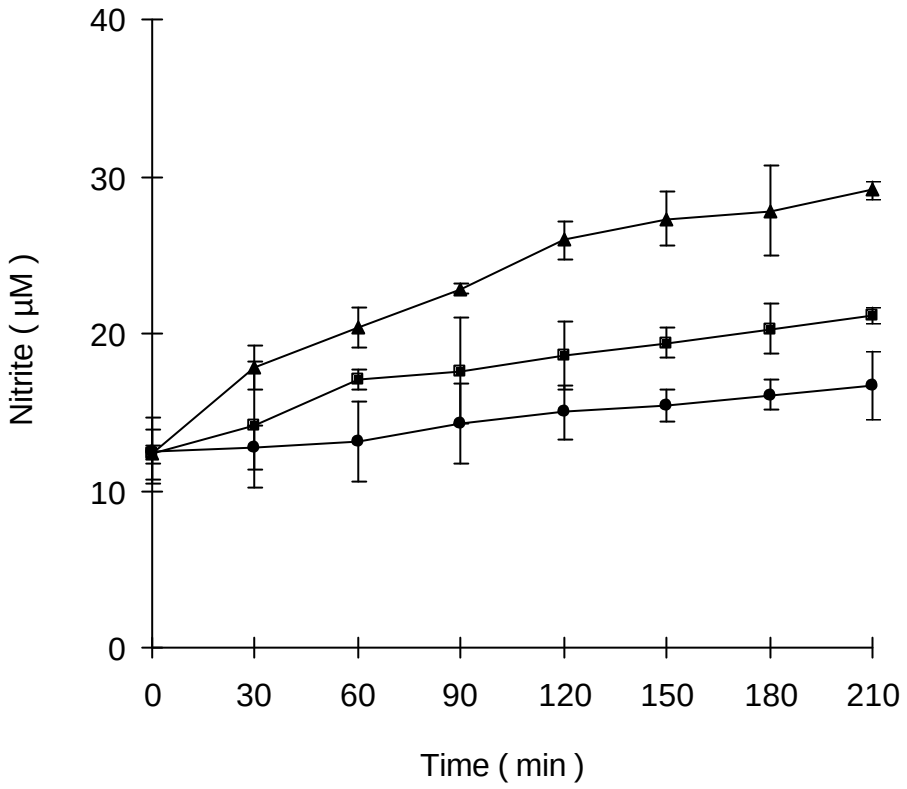


Figure 13. Time course of the formation of nitrite from the reaction of S-nitroso-N-acetylcysteine (NACysNO) and diethanolamine (DEA). Reaction mixtures contained 1.25 mM NACysNO and 1.25 mM DEA in 10 mM phosphate buffer, pH 7.0 at 37° C (■), 1.25 mM NACysNO and 2.5 mM DEA (◆) and 1.25 mM NACysNO control (●). Results are given as means of three experiments ± SD.

Table 3. Transnitrosation from S-nitrosothiols to diethanolamine yielding N-nitrosodiethanolamine.

		Time								
		0 min			60 min			210 min		
Mixture	(molar ratio)	RSNO (mM)	N-DEA (μM)	NO_2^- (μM)	RSNO (mM)	N-DEA (μM)	NO_2^- (μM)	RSNO (mM)	N-DEA (μM)	NO_2^- (μM)
CysNO/DEA	(1:0)	1.19 ± 0.02	-	36.2 ± 3.2	0.36 ± 0.05	-	291 ± 3.3	n.m.	n.m.	n.m.
	(1:1)	1.01 ± 0.11	7.0 ± 1.1	31.9 ± 6.5	0.21 ± 0.04	48.5 ± 2.0	489 ± 14.7	n.m.	n.m.	n.m.
	(1:2)	1.05 ± 0.05	28.4 ± 0.2	32.3 ± 3.6	-	138.0 ± 2.9	n.m.	n.m.	n.m.	n.m.
CGNO/DEA	(1:0)	1.13 ± 0.15	-	36.3 ± 5.8	0.25 ± 0.03	-	238 ± 11.3	n.m.	n.m.	n.m.
	(1:1)	1.09 ± 0.03	0.5 ± 0.10	36.2 ± 4.5	0.09 ± 0.01	38.0 ± 1.2	538 ± 9.8	n.m.	n.m.	n.m.
	(1:2)	1.03 ± 0.03	1.4 ± 0.00	36.2 ± 7.6	-	89.0 ± 8.4	n.m.	n.m.	n.m.	n.m.
HCysNO/DEA	(1:0)	1.18 ± 0.04	-	10.2 ± 3.3	1.17 ± 0.06	-	16.7 ± 1.7	1.18 ± 0.03	-	18.8 ± 1.2
	(1:1)	1.19 ± 0.04	-	8.9 ± 1.1	1.18 ± 0.05	0.40 ± 0.06	26.6 ± 3.4	1.16 ± 0.03	2.09 ± 0.24	45.8 ± 3.6
	(1:2)	1.18 ± 0.12	-	14.8 ± 2.9	1.15 ± 0.13	1.71 ± 0.60	35.9 ± 2.1	1.12 ± 0.05	4.40 ± 1.02	79.9 ± 5.0
GSNO/DEA	(1:0)	1.23 ± 0.03	-	10.5 ± 1.3	1.22 ± 0.06	-	13.4 ± 1.3	1.22 ± 0.02	-	20.3 ± 3.6
	(1:1)	1.19 ± 0.04	-	10.3 ± 1.4	1.19 ± 0.01	0.53 ± 0.14	16.7 ± 2.4	1.18 ± 0.01	0.93 ± 0.09	31.2 ± 1.9
	(1:2)	1.18 ± 0.05	-	11.0 ± 1.3	1.15 ± 0.03	0.88 ± 0.22	17.8 ± 2.0	1.14 ± 0.02	1.74 ± 0.35	43.5 ± 1.6
NACysNO/DEA	(1:0)	1.25 ± 0.01	-	12.5 ± 2.1	1.25 ± 0.02	-	13.1 ± 1.4	1.25 ± 0.01	-	16.7 ± 2.2
	(1:1)	1.25 ± 0.03	-	12.3 ± 1.6	1.24 ± 0.02	0.15 ± 0.01	17.1 ± 1.4	1.24 ± 0.01	0.43 ± 0.07	21.1 ± 1.2
	(1:2)	1.24 ± 0.02	-	12.3 ± 0.6	1.15 ± 0.03	0.25 ± 0.08	20.4 ± 1.1	1.23 ± 0.01	1.22 ± 0.04	29.2 ± 0.5

The mixtures contained 1.25 mM S-nitrosothiols (RSNO) in 10 mM phosphate buffer, pH 7.0, at 37°C, either no DEA (1:0 molar ratio), 1.25 mM DEA (1:1 molar ratio) or 2.5 mM DEA (1:2 molar ratio). RSNOs and N-DEA concentrations were determined by HPLC. Results represent means of three experiments (± SD), -: below detection limit; n.m.: not measured.

DEA, diethanolamine; N-DEA, N-nitrosodiethanolamine; CysNO, S-nitrosocysteine; CGNO, S-nitrosocysteinylglycine; HCysNO, S-nitrosohomocysteine; GSNO, S-nitrosoglutathione; NACysNO, S-nitroso-N-acetylcysteine.

3. 6. Nitrosation of diethanolamine with peroxynitrite

For comparison to S-nitrosocompounds, the nitrosating activity of peroxynitrite was investigated, measuring the yield of N-nitrosodiethanolamine when diethanolamine was incubated with 1 or 2 mM peroxynitrite for 15 min at pH 7 and 37°C. At a ratio of 1 mM diethanolamine and 1 mM peroxynitrite the yield of N-nitrosodiethanolamine was about 3.5 µM (transnitrosation efficacy of 0.35%). The yield of N-nitrosodiethanolamine increased to 46 µM with 2 mM peroxynitrite (transnitrosation efficay of 2.3%). The reaction was completed within seconds (table 4).

Table 4. Formation of N-nitrosodiethanolamine from the reaction of diethanolamine and peroxynitrite in 10 mM phosphate buffer pH 7.0, at 37°C.

	N-DEA (µM)	
Time	1 mM peroxynitrite:1 mM DEA	2 mM peroxynitrite: 1mM DEA
0 min	3.63 ± 0.4	45.8 ± 1.2
15 min	3.30 ± 0.6	46.6 ± 0.8

3.7. Sulfur- to- sulfur transnitrosation

The exchange of NO between thiol groups, involving cysteine, homocysteine and the respective S-nitrosothiols is shown in figures 14 and 15. CysNO was incubated together with homocysteine and the decay of CysNO as well as the formation of HCysNO were determined as a function of time (figure 14). Figure 15 shows the results of a similar experiment using HCysNO and cysteine as reactants. The transnitrosation was determined by co-incubation of equimolar amounts of the S-nitroso compounds (100 µM) and thiols (100 µM) (figure 14 and 15). The transnitrosation between S-nitrosocysteine and homocysteine and S-nitrosohomocysteine and cysteine is shown in reaction 1 and 2.

CysNO + HCys ⟶ HCysNO + Cys (1)

HCysNO + Cys ⟶ CysNO + HCys (2)

3.8. S-Nitrosocysteine stability in phosphate buffer containing different concentrations of homocysteine

It was suggested that S-nitroso compounds are intermediate storage and transport forms of nitric oxide. Factors that influence the release of NO from S-nitroso compounds or have impact on their stability may be of biological importance. The following experiments were performed to investigate the fate of the NO-group using S-nitrosocysteine as a donor molecule. When S-nitrosocysteine was alone incubated in 10 mM phosphate buffer, pH 7.0 at 37°C, it decomposed rapidly and the NO-group was completely lost after 20 min of incubation. The apparent half-life of S-nitrosocysteine was much larger when 100 µM cysteine where present in the incubation mixture. At 20 min almost 80% of the S-nitrosocysteine was still present (figure 16). This is probably due to S-to-S transnitrosation reactions between S-

nitrosocysteine and cysteine which are competitive to the denitrosation of S-nitrosocysteine and conserve the NO in an active S-nitroso structure.

The presence of other thiols which may be involved in additional transnitrosation reactions may further modify the half-life of NO bound as a nitroso-thiol. Especially thiols that form rather stable S-nitroso derivatives may significantly influence the NO-flux and might even be nitric oxide traps. In order to investigate this aspect further experiments were performed in which increasing amounts of homocysteine were added to the incubation mixture. In the presence of S-nitrosocysteine, formation of the relatively stable S-nitrosohocysteine is an additional competitive pathway to denitrosation (figure 17). This leads to increased half-life or "availability" of total S-nitroso compounds in the mixture. With increasing amounts of homocysteine in the mixture the half-life of NO conserved or trapped as S-nitroso derivative increases. With 50 µM homocysteine, no loss of S-nitrosothiols is observed after 40 min of incubation (figure 18).

Such an influence on the stability of S-nitroso compounds was still observed when BSA as a protein and possible nitrosation target was additionally added to the mixture (figure 19). The impact of increasing amounts of homocysteine on the apparent half-life of total S-NO was still detectable. In the presence of 50 µM homocysteine there was no loss of total S-NO after 20 min of incubations, whereas 30% of the total S-nitrosothiols were lost at that time point when no homocysteine present.

Stability effects on the amount of total S-NO mediated by increasing amounts of homocysteine were also found when human serum was used as an incubation medium. The effects were less preserved which might be due to the presence of a number of other compounds that have impact on the stability and transnitrosation efficacy. However, again with 50 µM homocysteine (figure 20) the loss of total S-

nitroso-compound in serum was slower as compared to mixtures with amounts or no homocysteine. For better comparison the data were repeated with diluted serum; here the effects are more obvious (figure 20).

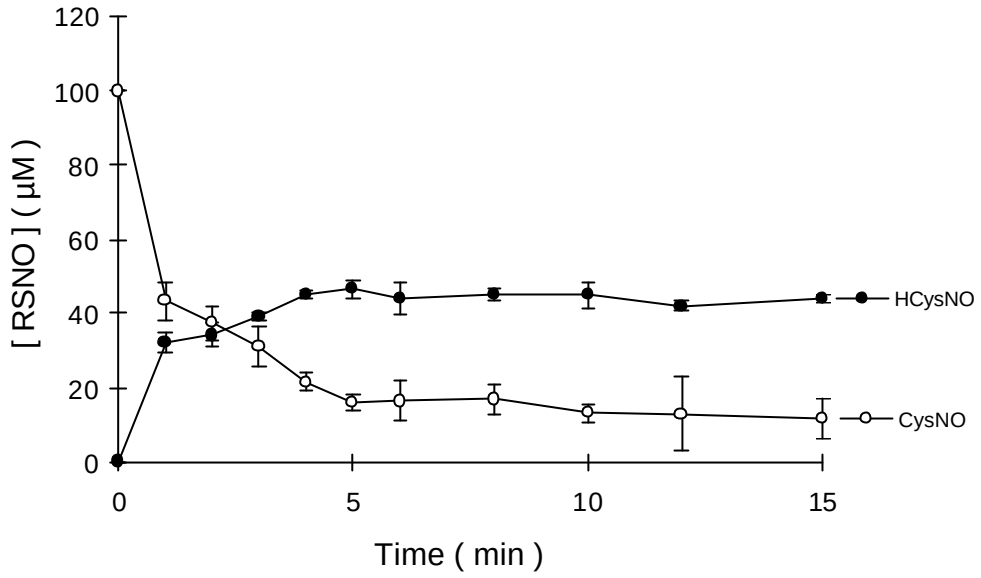


Figure 14 . Transnitrosation between S-nitrosocysteine (CysNO) and homocysteine (HCys). CysNO (100 µM) was incubated with HCys (100 µM) and both the decay of CysNO and the formation of HCysNO were monitored by HPLC as a function of time. Data represent means ± SD (n=3)

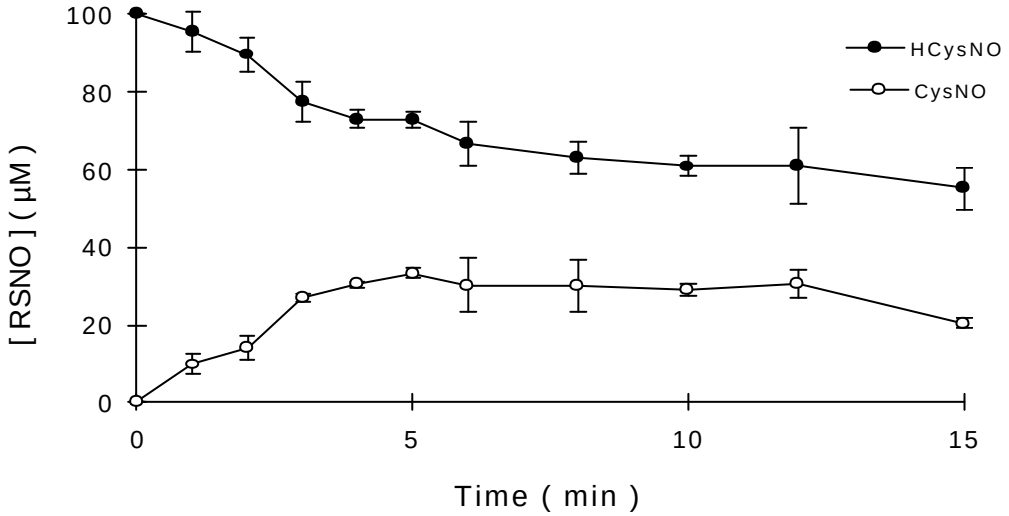


Figure 15. Transnitrosation between S-nitrosohomocysteine (HCysNO) and cysteine (Cys). HCysNO (100 µM) was incubated with Cys (100 µM) and both the decay of HCysNO and the formation of CysNO were monitored by HPLC as a function of time. Data represent means ± SD (n=3).

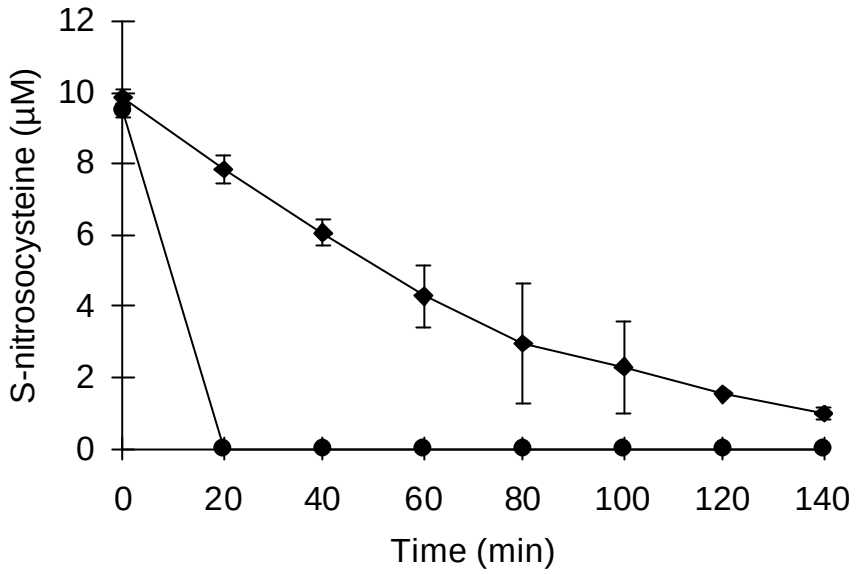


Figure 16. Stability of S-nitrosocysteine in phosphate buffer. 10 µM S-nitrosocysteine was incubated with (◆) or without 100 µM cysteine (●) in 10 mM phosphate buffer pH 7.0, at 37°C. Data represent means ± SD (n=3).

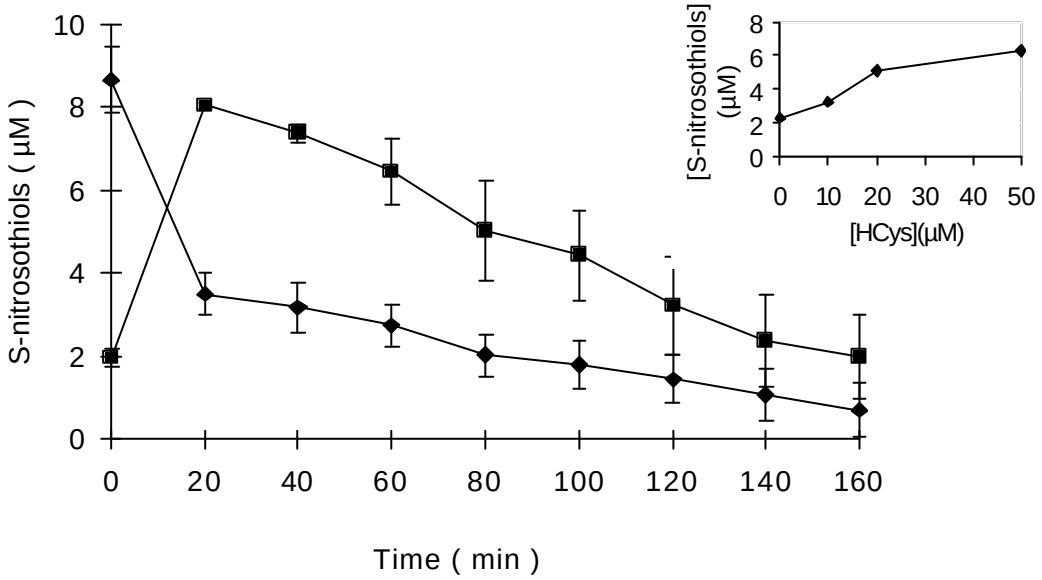


Figure 17. Stability of S-nitrosocysteine in the presence of homocysteine. 10 µM S-nitrosocysteine was incubated in 10 mM phosphate buffer pH 7.0, at 37°C contained 100 µM cysteine and 50 µM homocysteine, S-nitrosocysteine (◆), S-nitrosohomocysteine (■). Data represent means ± SD (n=3). Inset to (fig 17), showed the total concentration of S-nitrosothiols (in the presence of 100 µM cysteine) after 100 min incubation of 10 µM S-nitrosocysteine with various concentration of homocysteine.

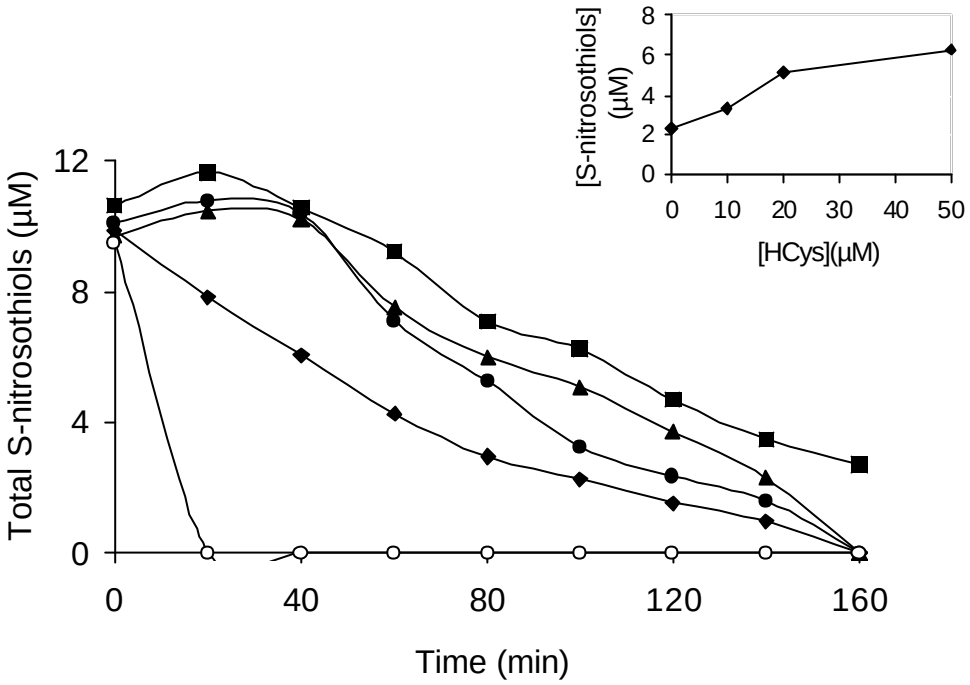


Figure 18: Stability of S-nitroso-compounds in the presence of homocysteine incubated in 10 mM phosphate buffer pH 7.0, at 37°C. Reaction mixture contained 10 µM S-nitrosocysteine (O), 10 µM S-nitrosocysteine /100µM cysteine (◆), 10 µM S-nitrosocysteine /100µM cysteine/10 µM homocysteine (●), 10 µM S-nitrosocysteine /100µM cysteine/20 µM homocysteine (**?**), 10 µM S-nitrosocysteine /100µM cysteine/50 µM homocysteine (■). Inset to (fig 18), data taken from figure 18 and plotted as total concentration of S-nitrosothiols against concentration of homocysteine (in the presence of 100 µM cysteine) after 100 min incubation. Data represent mean of three experiments.

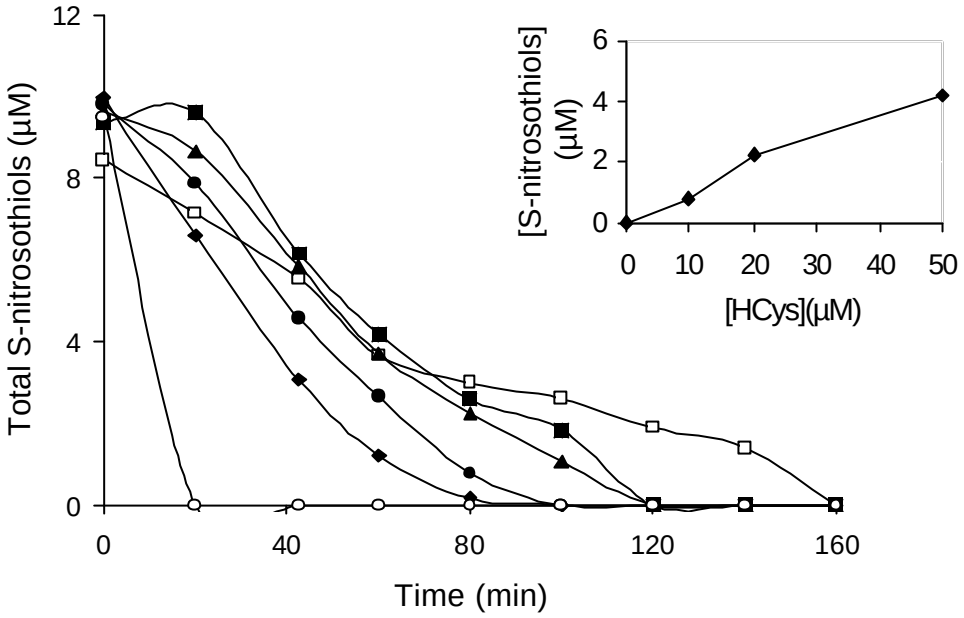


Figure 19: Stability of S-nitroso-compounds in the presence of homocysteine and BSA incubated in 10 mM phosphate buffer pH 7.0, at 37°C. Reaction mixture contained 10 µM S-nitrosocysteine (O), 10 µM S-nitrosocysteine /100 µM cysteine /BSA (4 mg/ml) (◆),10 µM S-nitrosocysteine /100 µM cysteine/ BSA (4 mg/ml) and10 µM homocysteine (●),10 µM S-nitrosocysteine /100 µM cysteine/ BSA (4 mg/ml) and 20 µM homocysteine (**?**),10 µM S-nitrosocysteine /100 µM cysteine/ BSA (4 mg/ml) and 50 µM homocysteine (■),10 µM S-nitrosocysteine / BSA (4 mg/ml), (?). Inset to (fig 19), data taken from figure19 and plotted as total concentration of S-nitrosothiols against concentration of homocysteine (in the presence of 100 µM cysteine and BSA) after 80 min incubation. Data represent mean of three experiments.

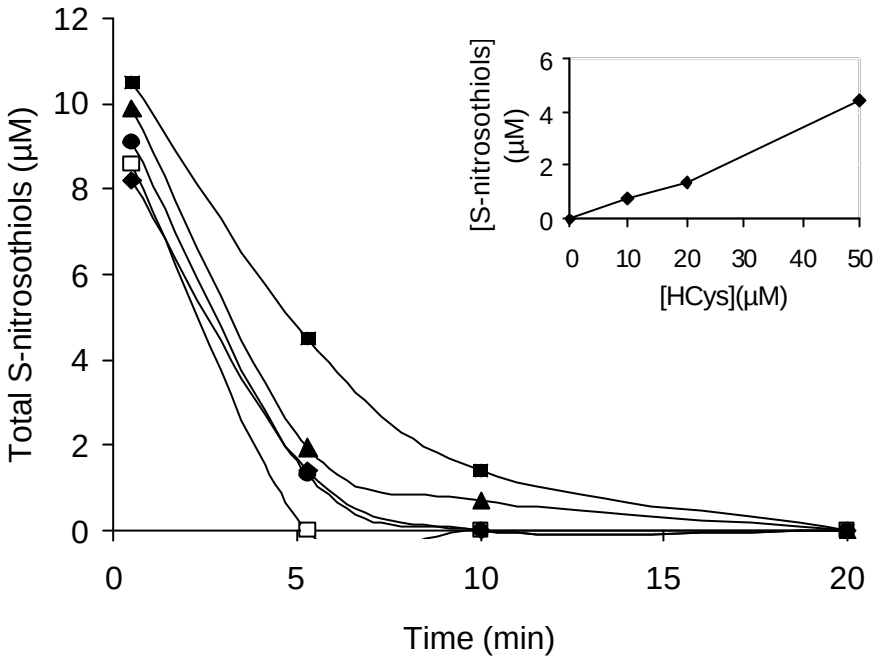


Figure 20: Stability of S-nitroso-compounds in human serum in the presence of homocysteine. Reaction mixture contained 1 ml human serum was incubated at 37°C with 10 µM S-nitrosocysteine ( ), 10 µM S-nitrosocysteine /100µM cysteine (◆),10 µM S-nitrosocysteine /100µM cysteine/10 µM homocysteine (●),10 µM S-nitrosocysteine /100µM cysteine/20 µM homocysteine (**?**), and 10 µM S-nitrosocysteine /100µM cysteine/50 µM homocysteine (■). Inset to (fig 20), data taken from figure 20 and plotted as total concentration of S-nitrosothiols against concentration of homocysteine (in the presence of 100 µM cysteine) after 5 min incubation. Data represent mean of three experiments.

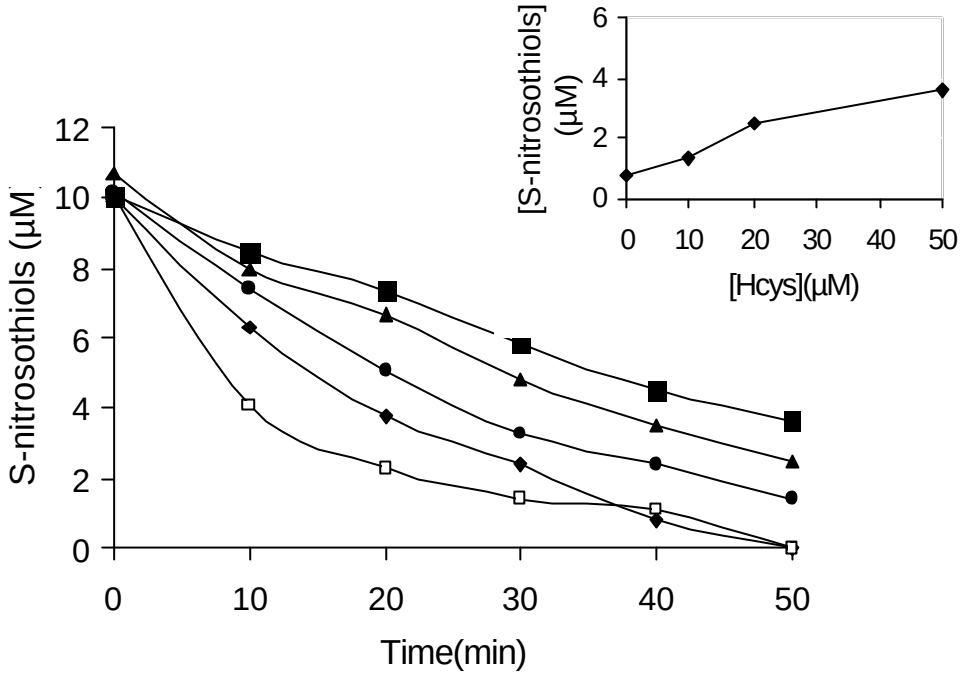


Figure 21: Stability of S-nitroso-compounds in human serum. Reaction mixture contained human serum diluted 1:10 in 10 mM phosphate buffer pH 7.0 at 37°C was incubated with 10 µM S-nitrosocysteine ( ), 10 µM S-nitrosocysteine /100µM cysteine (◆), 10 µM S-nitrosocysteine /100µM cysteine/10 µM homocysteine (●), 10 µM S-nitrosocysteine /100µM cysteine/20 µM homocysteine (?), and 10 µM S-nitrosocysteine /100µM cysteine/50 µM homocysteine (■). Inset to (fig 21): Data taken from figure 21 and plotted as total concentration of S-nitrosothiols against concentration of homocysteine (in the presence of 100 µM cysteine) after 100 min incubation. Data represent mean of three experiments.

3.9. Purification of microsomal glutathione S-transferase.

For the purification of microsomal glutathione S-transferase from pig kidney, hydroxyapatite and CM-Sepharose chromatography was applied. Two peaks of glutathione S-transferases activity with GSH as substrate were obtained with hydroxyapatite column chromatography (figure 22) whereas only one peak of glutathione S-transferase activity towards the substrate N-acetyl-L-cysteine was obtained in this system (figure 22). The hydroxyapatite peak-I and peak-II fractions of kidney microsomes were applied to SDS-polyacrylamide gel electrophoresis (15%) to see the separation of membrane bound cytosolic GSTs from microsomal forms (figure 24).

When the hydroxyapatite peak-II fractions were collected and chromatographed on a CM-Sepharose column, only one peak was obtained and exhibiting transferase activity (figure 23). The active fractions were collected and applied to SDS-polyacrylamide gel electrophoresis (15%). Only one protein band was obtained after silver staining (figure 25). As expected from its substrate specificity toward N-acetyl-L-cysteine, the glutathione S-transferase in the hydroxyapatite peak-II fractions of kidney microsomes purified by CM-Sepharose, had a molecular weight of 17.3 kDa as shown in figures (25 & 26B) .

The hydroxyapatite peak-I fractions were further purified by S-hexylglutathione affinity chromatography and the active fractions enzyme from the affinity chromatography column were collected and exhibited only one band on SDS-polyacrylamide gel electrophoresis (15%)(figure 26A); its apparent molecular weight was calculated as 26 kDa, as reported for other alpha-class glutathione S-transferases (Kamisaka, et al. 1975). This form was compared with glutathione S-transferases purified from pig kidney cytosol by S-hexylglutathione affinity chromatography and seemed to be a form of the membrane-bound cytosolic glutathione S-transferases (figure 26A).

To compare the two forms of the glutathione S-transferases purified from pig kidney microsomes by S-hexylglutathione and CM-Sepharose chromatography, the two forms were applied to SDS-polyacrylamide gel electrophoresis and it was found that both of them migrated with different MW, one form with MW of 26 kDa and the other one with 17.3 kDa (figure 26B).

3.10. Properties of pig kidney microsomal glutathione S-transferase.

As shown in figure 20 from the two glutathione S-transferases isolated from kidney microsomes, only hydroxyapatite peak-II showed activity towards N-acetyl-L-cysteine as substrate. To compare the pig kidney microsomal glutathione S-transferase with rat liver microsomal glutathione S-transferase, the rat liver microsomal glutathione S-transferase was isolated from rat liver microsomes as described before (see 2.17). Both enzymes were studied with respect to the influence of NO donor compounds such as S-nitrosothiols and peroxynitrite and alkylating agent such as N-ethylmaleimide on their activity. Treatment of the pig kidney microsomal glutathione S-transferase with NEM resulted in 14% inhibition, whereas, the rat liver microsomal glutathione S-transferase showed a 5.4-fold activation (table 5). Exposure of the pig kidney and rat liver microsomal glutathione S-transferase to peroxynitrite resulted in 23% inhibition and 120% activation, respectively (table 5). Incubation of pig kidney microsomal glutathione S-transferase with S-nitrosocysteine led to 9% inhibition, while the rat liver microsomal glutathione S-transferase showed 2-fold activation (table 5). It is remarkable that in kidney microsomal glutathione S-transferase no stimulation with NEM could be seen.

3.11. Amino acid sequence of pig kidney microsomal glutathione S-transferase

The purified microsomal glutathione S-transferases from pig kidney and liver microsomes were sequenced. As shown in table 6 the first 10 amino acids in the sequence of pig kidney and liver microsomal glutathione S-transferase are similar. A comparison between rat liver and pig microsomal glutathione S-transferase, showed differences at positions 4 and 5; and homology was more than 80%.

3.12. Nitration of tyrosine residues of glutathione S-transferase

Pig kidney microsomal glutathione S-transferase and rat liver cytosolic glutathione S-transferase showed different sensitivities towards $ONOO^-$ and S-nitrosothiols as shown in figures 27, 30 and table 6. Both were treated with peroxynitrite and subjected to SDS-polyacrylamide gel electrophoresis and blotted towards monoclonal anti-3-nitrotyrosine antibodies. Only one band showed nitrotyrosine labeling and verified the nitration of microsomal and cytosolic glutathione S-transferase (figure 28 and 29).

3.13. Influence of peroxynitrite on glutathione S-transferase activity.

The effect of $ONOO^-$ on glutathione S-transferase activity was analysed. As shown in figure (27), the rat liver cytosolic glutathione S-transferase activity was found to be inhibited by $ONOO^-$ in a concentration-dependent manner. Incubation of the rat liver cytosolic glutathione S-transferase with 50 µM $ONOO^-$ inhibited the enzyme by 50% (figure 27), while rat liver microsomal glutathione S-transferase was insensitive (table 5). When pig kidney microsomal glutathione S-transferase treated with peroxynitrite, the enzyme was sensitive and showed 23% inhibition (table 5).

3.14. Effect of S-nitrosothiols on glutathione S-transferase activity

Rat liver cytosolic glutathione S-transferase was initially treated with S-nitrosocysteine, or S-nitrosohomocysteine in presence or absence of cysteine and glutathione S-transferase activity was followed. The enzyme used was > 95% pure as determined with SDS–polyacrylamide gel electrophoresis (figure 24 and 25). Incubation of rat liver glutathione S-transferase with S-nitrosocysteine resulted in a concentration-dependent loss of glutathione S-transferase activity (figure 30), whereas no influence on the rat liver microsomal glutathione S-transferase activity was shown (table 5). Comparing to S-nitrosocysteine, S-nitrosohomocysteine revealed the lowest action e.g. 5 mM S-nitrosohomocysteine inhibited rat liver cytosolic glutathione S-transferase by 6%, whereas 5 mM S-nitrosocysteine provided 67% inhibition of cytosolic glutathione S-transferase (figure 30). Addition of cysteine to S-nitrosohomocysteine led to inhibition of the rat liver cytosolic glutathione S-transferase activity by 17%, whereas only 6% inhibition was achieved by S-nitrosohomocysteine (figure 30).

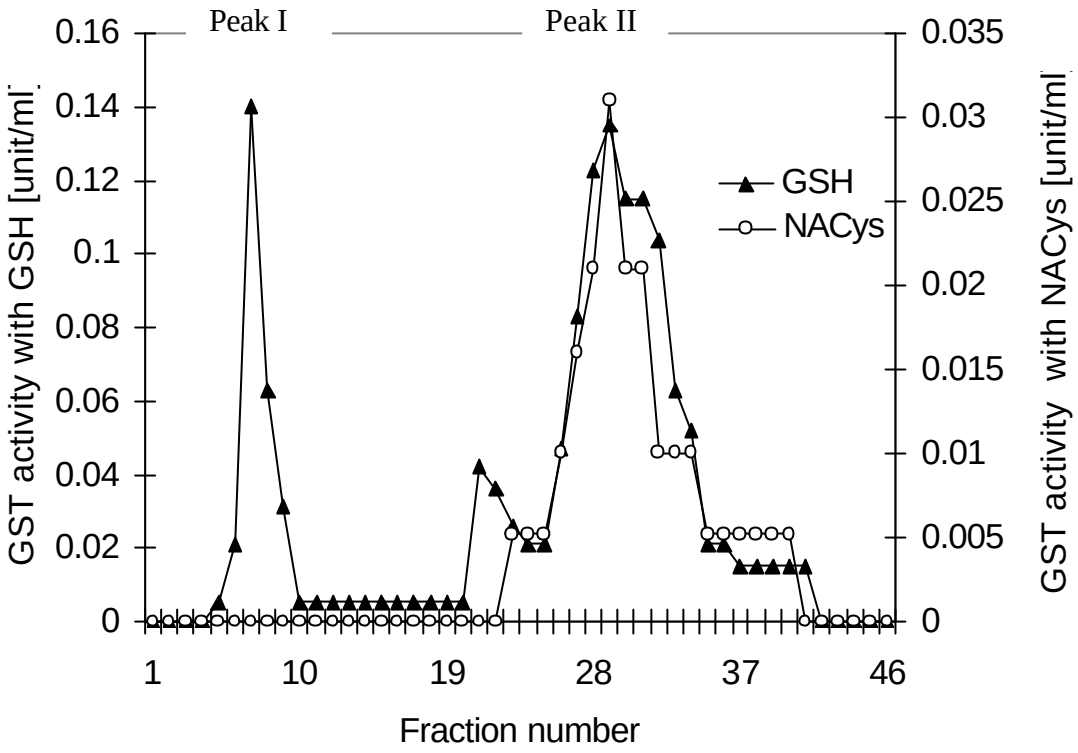


Figure 22: Elution pattern of pig kidney microsomal glutathione S-transferases activity from hydroxyapatite column chromatography. The glutathione S-transferases activity of each fraction was measured in the presence of glutathione (GSH) or N-acetylcysteine (NACys) and CDNB.

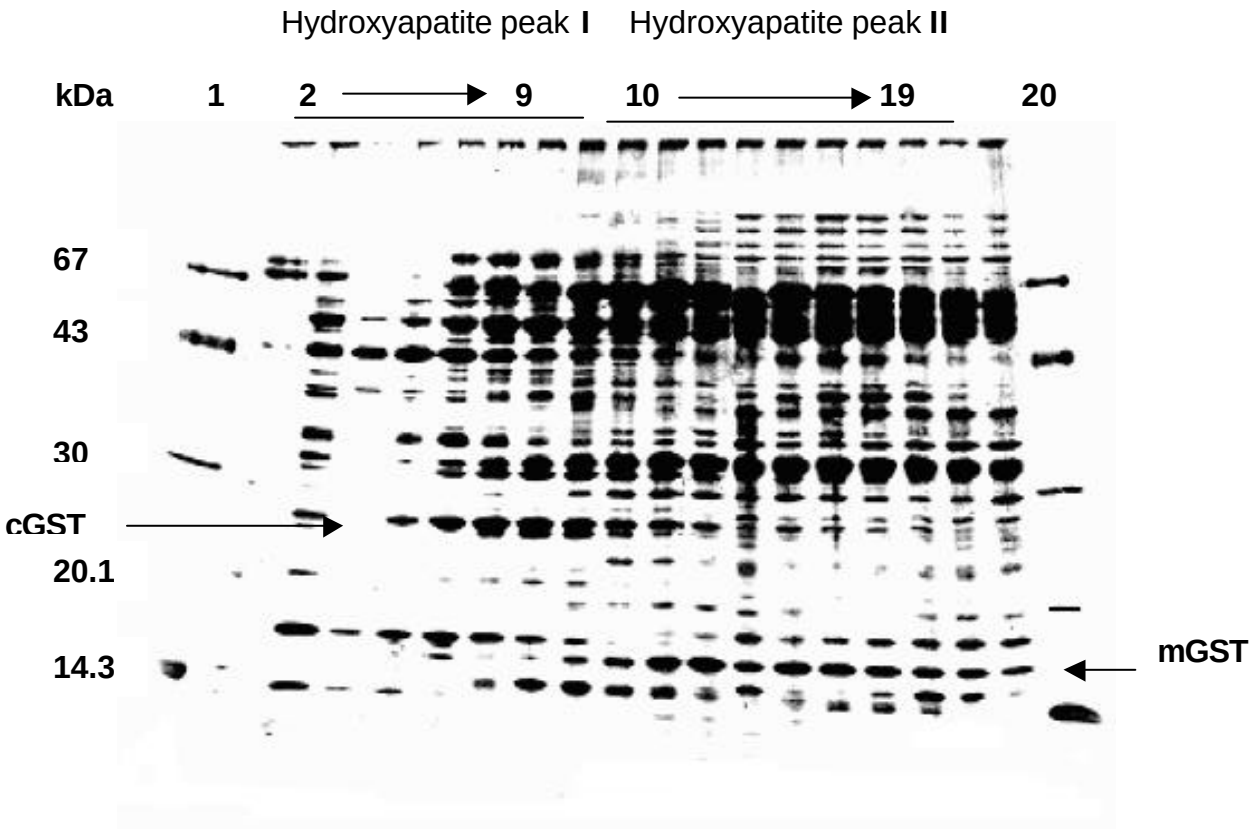


Figure 23: SDS-PAGE of glutathione S-transferase fractions from pig kidney microsomes eluted from hydroxyapatite chromatography. Lane 1 and 20: low molecular size standard, lane 2 - 9: pig kidney glutathione S-transferase (active fraction from peak I) (see fig.18), lane 10 - 19: pig kidney glutathione S-transferase (active fraction from peak II). SDS-PAGE was performed in a 15% acrylamide gel and stained with silver nitrite.

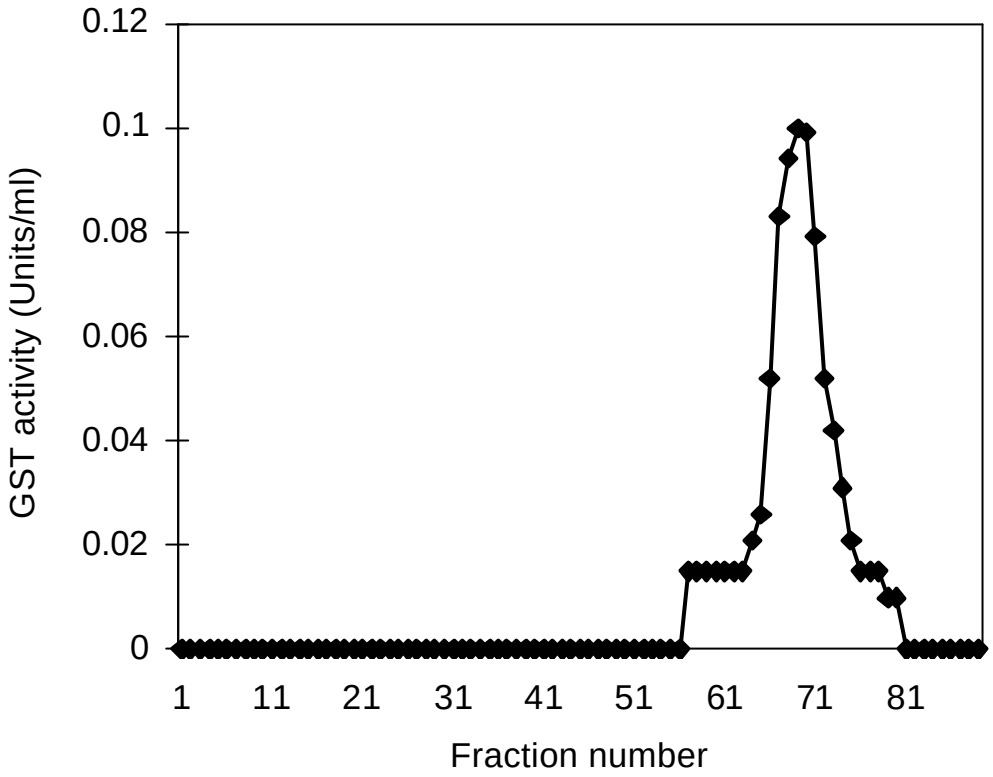


Figure 24: Elution pattern of pig kidney microsomal glutathione S-transferase (hydroxyapatite peak II) activity from CM-Sepharose column chromatography. The glutathione S-transferases activity of each fraction (1.6 ml) was measured in the presence of glutathione (GSH) and CDNB.

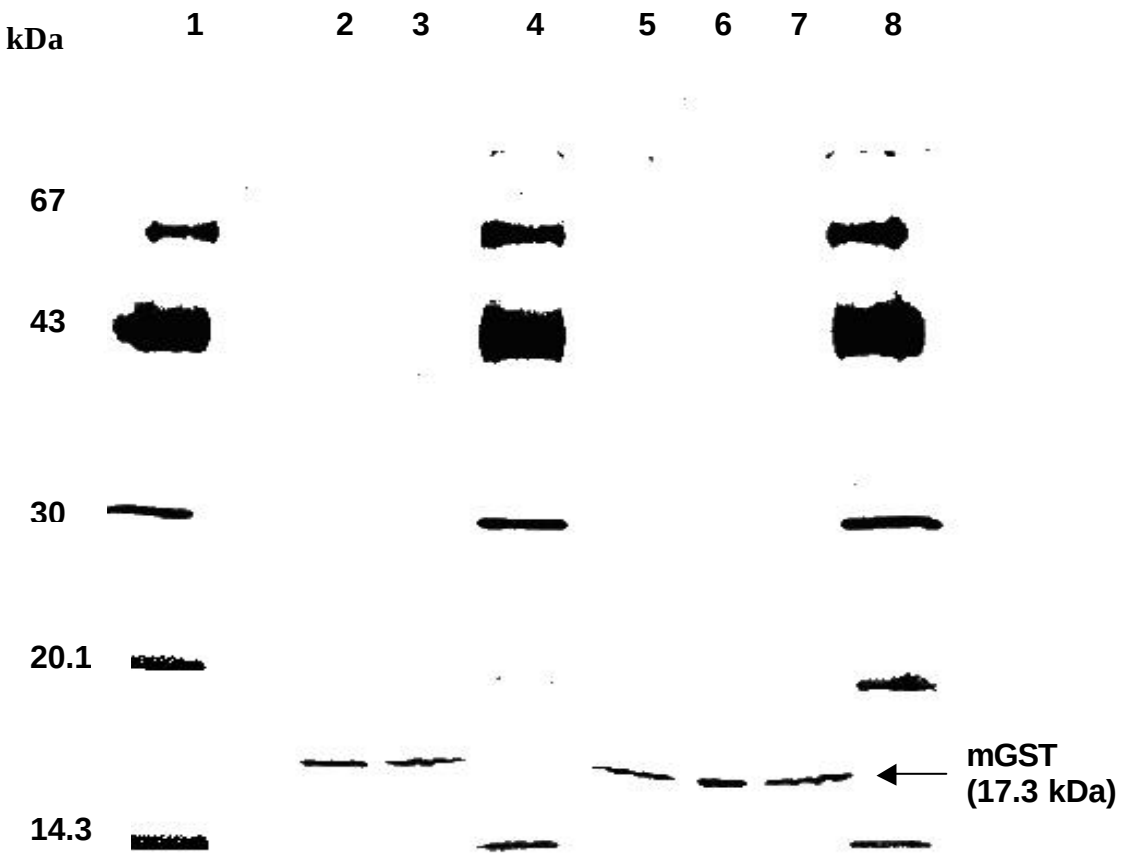


Figure 25: SDS-PAGE of purified microsomal glutathione S-transferase from pig kidney and liver microsomes. Lane 1, 4 and 8: low molecular size standard, lane 2 and 6: pig kidney microsomal glutathione transferase1 purified by CM-Sepharose, lane 3 and 7: Pig liver microsomal glutathione transferase1 purified by CM-Sepharose and lane 5: mixture of both. SDS-PAGE was performed in a 15% acrylamide gel and stained with silver nitrite.

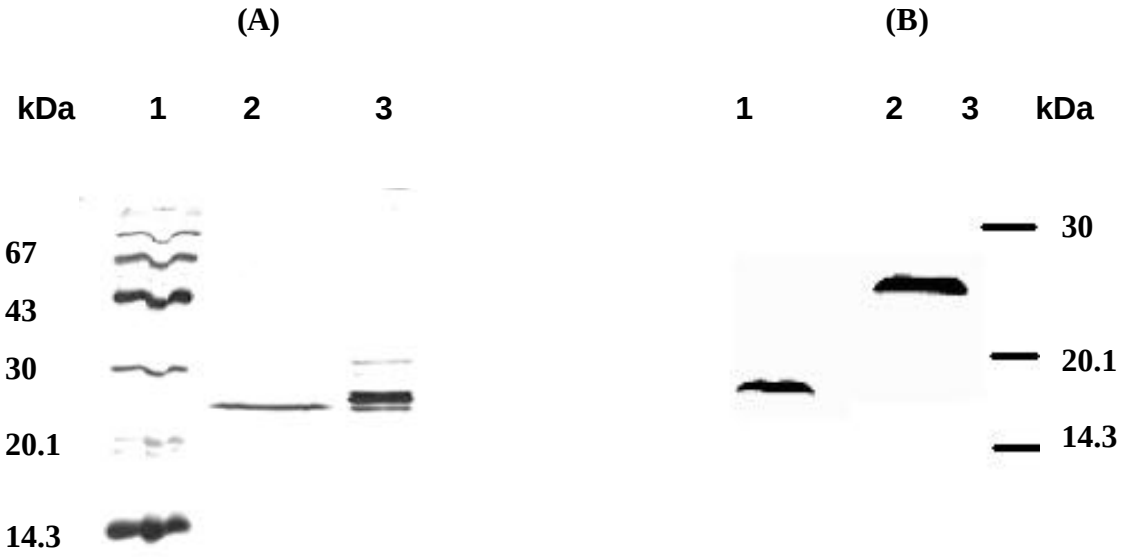


Figure 26A. SDS-PAGE of glutathione S-transferase purified from pig kidney microsomes and cytosol by S-hexylglutathione chromatography. Lane 1: low molecular size standard, lane 2: pig kidney glutathione S-transferase purified from hydroxyapatite peak I (see fig. 18) by S-hexylglutathione chromatography, lane 3: cytosolic glutathione S-transferases purified from pig kidney cytosol by S-hexylglutathione chromatography. B: lane 1: pig kidney microsomal glutathione transferase1 (hydroxyapatite peak II) purified by CM-Sepharose, lane 2: pig kidney glutathione S-transferase purified from hydroxyapatite peak I and lane 3: low molecular size standard. SDS-PAGE was performed in a 15% acrylamide gel and stained with silver nitrite.

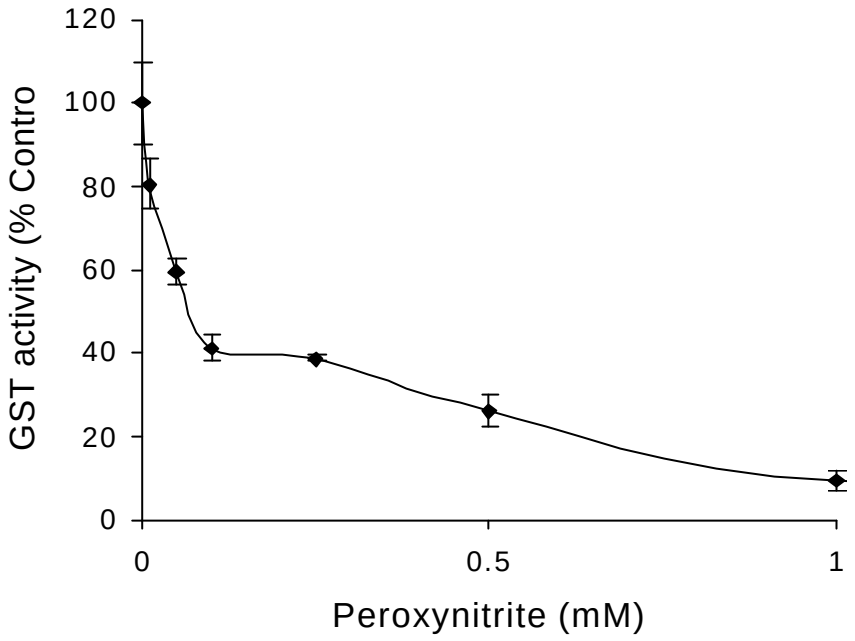


Figure 27. Effect of peroxynitrite on rat liver cytosolic glutathione S-transferase activity. Peroxynitrite (0 -1 mM in 100 mM NaOH) was added to GST (9 units/mg/ml in 100 mM phosphate buffer pH 7.0, 0.1 mM DTPH at 25°C). The pH of the mixture was not significantly altered by addition of peroxynitrite. GST activity was determined after 10 min incubation. Results represent mean ± SD. (n=3).

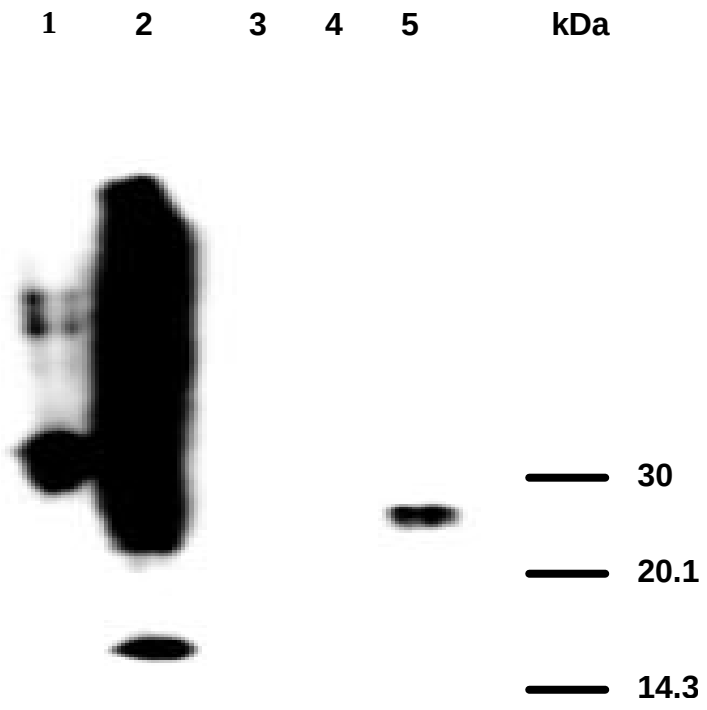


Figure 28: Western blot analysis performed using monoclonal anti-3-nitrotyrosine antibody. Nitration of tyrosine residues of rat liver cytosolic and pig kidney microsomal glutathione S-transferases by peroxynitrite. Protein nitration was examined using a monoclonal anti-3-nitrotyrosine antibody. GAPDH treated with 80 µM peroxynitrite (Lane 1), microsomal glutathione S-transferases eluted from hydroxyapatite chromatography (peak II, see fig 18) treated with 1 mM peroxynitrite) (lane 2), lane 3: microsomal glutathione S-transferases exposed to 1 mM decomposed peroxynitrite (lane 3), microsomal glutathione S-transferases (control) (lane 4) and rat liver cytosolic glutathione S-transferases treated with 1 mM peroxynitrite (lane 4).

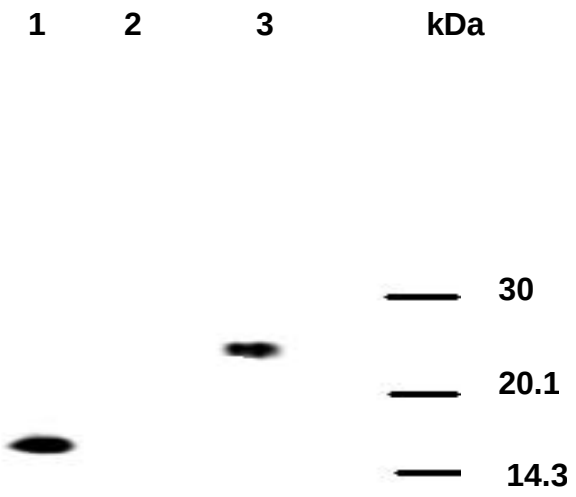


Figure 29. Western blot analysis performed using monoclonal anti-3-nitrotyrosine antibody. Nitration of pig kidney microsomal glutathione S-transferase 1 by peroxynitrite. Protein nitration was examined using monoclonal anti-3-nitrotyrosine antibody. Enzyme was exposed to 1 mM peroxynitrite (lane 1),or to the predecomposed 1 mM peroxynitrite (lane 2) and rat liver cytosolic glutathione S-transferases exposed to 1 mM peroxynitrite (lane 3).

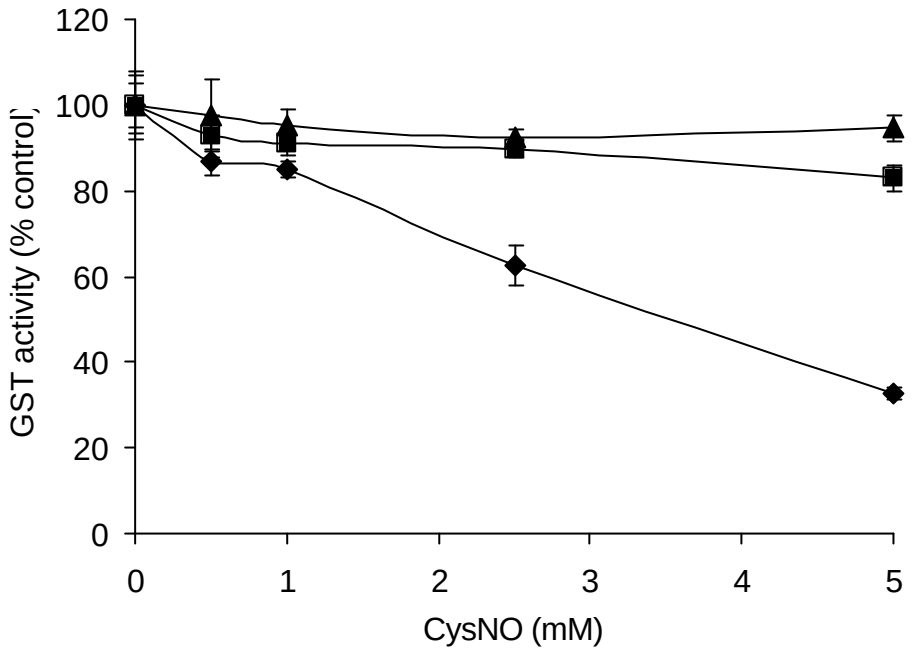


Figure 30. Effect of S-nitrosothiols on cytosolic glutathione S-transferase activity. S-nitrosocysteine (0–5.0 mM)(◆) was added to GST (9 unit/mg/ml) in 10 mM phosphate buffer pH 7.0, at 25°C) and vortex-mixed immediately. Activity was determined after 10 min incubation, GST activity in the presence of S-nitrosohomocysteine (0 –5.0 mM)(?), GST activity in the presence of S-nitrosohomocysteine (0 –5.0 mM) and 1 mM cysteine (■). Results represent mean ± SD (n=3).

Table 5: Microsomal glutathione S-transferase 1 activity

	Control (unit)	with NEM (unit)	Activation (%)
Rat liver mGST	0.39 ± 0.10	2.10 ± 0.4	538
Pig kidney mGST	0.22 ± 0.05	0.19 ± 0.1	86
	Control (unit)	with PN (unit)	
Rat liver mGST	0.39 ± 0.10	0.47 ± 0.2	120
Pig kidney mGST	0.22 ± 0.05	0.17 ± 0.3	77
	Control (unit)	with CysNO (unit)	
Rat liver mGST	0.39 ± 0.10	0.40 ± 0.3	102
Pig kidney mGST	0.22 ± 0.05	0.20 ± 0.1	91

NEM: N-Ethylmaleimide; PN: peroxynitrite; unit: µmol/min/mg

Table 6. Amino acid sequence of microsomal glutathione transferase.

Rat liver mGST	A D L K Q L M D N E	Morgenstern, *et. al.* 1985
Pig liver mGST	A D LT E LM D N E	EMBO-Data bank
Pig kidney mGST	A D L T E L M - N -	

6. References

Abramson S.B., Leszczynska-Piziak J., Yegudin J., *et al*. EDRFS (nitric oxide , *S*-nitrosothiols) agument human neutrophil chemotaxis while inhibiting superoxide anion generation : role of intracellular *S*-nitrosothiol formation. *Endothelium*.1993, 1, S29.

Airoldi L., Bonfanti M., Benfenati E., Tavecchia P., and Fanelli R. Identification of an acidic metabolite of N-nitrosodiethanolamine isolated from rat urine. *Biomed. Mass Spectrom.* 1983,10: 334-337.

Airoldi L., Bonfanti M., Fanelli R., Bove B., Benfenati E., and Gariboldi P., Identification of a nitrosamino aldehyde and a nitrosamino acid resulting from oxidation of N-nitrosodiethanolamine. *Chem. Biol. Interact.* 1984, 51:103-113.

Aleryani S., Milo E., Rose Y., and Kostka P. Superoxide- mediated decomposition of biological S-nitrosothiols. *J. Biol.Chem.* 1998, 273: 6041-6045.

Andersson C., Mosialou E., Weinander R., and Morgenstern R. In conjugation-dependent carcinogenicity and toxicity of foreign compounds (Anders, M. W., and Dekant, W., eds.) Academic Press, San Diego, CA. 1994, pp 19-35.

Andersson C, Piemonte F., Mosialou E., Weinander R., Sun TH., Lundqvist G., Adang AE., Morgenstern R. Kinetic studies on rat liver microsomal glutathione transferase: consequences of activation. *Biochim. Biophys. Acta*. 1995, 1247: 277-83.

Armstrong R.N. Structure, catalytic mechanism, and evolution of the glutathione transferases. *Chem. Res. Toxicol.* 1997, 10: 2-18.

Asahi M., Fujii J., Suzuki K., Seo H.G., Kuzuya T., Hori M., Tada M., Fujii S., Taniguchi N. Inactivation of glutathione peroxidase by nitric oxide. Implication for cytotoxicity. *J. Biol. Chem*. 1995, 270: 21035-9.

Bartsch H., Ohshima H., Pignatelli B. Inhibitors of endogenous nitrosation. Mechanisms and implications in human cancer prevention. *Mutat. Res.* 1988, 202: 307-24.

Barnett D.J., McAninly J. and Williams D.L.H. Transnitrosations between nitrosothiols and thiols. *J. Chem. Soc. Perkin Trans.* 1994, 2:1131-1133

Boushy C.J., Beresford S.A., Omenn G.S., and Motulsky A.G.A., Quantitative assessment of plasma homocysteine as a risk factor for vascular disease. *J. Am. Med. Assoc.* 1995, 274: 1049-1057.

Beckman J.S. Oxidative damage and tyrosine nitration from peroxynitrite. *Chem. Res. Toxicol.* 1996, 9: 836-44.

Beckman J.S., Beckman T.W., Chen J., Marshall P.A., Freeman B.A., Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. U. S. A.* 1990, 87: 1620-4.

Beckman J.S., and Koppenol W.H. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am. J. Physiol.* 1996, 271: C1424-37.

Berlett B.S., Levine R.L., Stadtman E.R. Carbon dioxide stimulates peroxynitrite-mediated nitration of tyrosine residues and inhibits oxidation of methionine residues of glutamine synthetase: both modifications mimic effects of adenlylation. *Proc. Natl. Acad. Sci. U. S. A.* 1998, 95: 2784-9.

Bredt D.S., and Snyder S.H. Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proc. Natl. Acad. Sci. U. S. A.* 1990, 87: 682-5.

Butz L**.W.,** and du Vigneaud V. The formation of a homologue of cystine by the decomposition of methionine with sulphuric acid. *J. Biol. Chem.* 1932, 99: 135-42.

Buchczyk D.P., Briviba K., Hartl F.U., Sies H. Responses to peroxynitrite in yeast: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a sensitive intracellular target for nitration and enhancement of chaperone expression and ubiquitination. *Biol. Chem.* 2000, 381: 121-6.

Butler A.R., and Rhodes P. Chemistry, analysis and biological roles of S-nitrosothiols. *Anal.Biochem.* 1997, 249: 1-9

Cassina A, and Radi R. Differential inhibitory action of nitric oxide and peroxynitrite on mitochondrial electron transport. *Arch. Biochem. Biophys.* 1996, 328: 309-16.

Chasseaud L.F. The role of glutathione and glutathione S-transferases in the metabolism of chemical carcinogens and other electrophilic agents. *Adv. Cancer Res.* 1979, 29: 175-274.

Clancy R.M., Levartovsky D., Leszczynska-Piziak J., Yegudin J., Abramson S.B. Nitric oxide reacts with intracellular glutathione and activates the hexose monophosphate shunt in human neutrophils: evidence for S-nitrosoglutathione as a bioactive intermediary. *Proc. Natl. Acad. Sci. U. S. A.* 1994, 91: 3680-4.

Clark A.G., Debnam P. Inhibition of glutathione S-transferases from rat liver by *S*-nitroso-L-glutathione. *Biochem Pharmacol.* 1988, 37:3199-201

Crow J.P., Beckman J.S., McCord J.M. Sensitivity of the essential zinc-thiolate moiety of yeast alcohol dehydrogenase to hypochlorite and peroxynitrite. *Biochemistry.* 1995, 34: 3544-52.

Crow J.P., Ye YZ., Strong M., Kirk M., Barnes S., Beckman J.S. Superoxide dismutase catalyzes nitration of tyrosines by peroxynitrite in the rod and head domains of neurofilament-L. *J. Neurochem.* 1997, 69: 1945-53.

DeJong J., Morgenstern R., Jörnvall H., Depierre J.W., and Tu C.P.D. Gene expression of rat and human microsomal glutathione S-transferases. *J. Biol. Chem.* 1988, 263: 8430-8436.

Denninger J.W., and Marletta M.A. Guanylate cyclase and the NO/cGMP signaling pathway. *Biochim Biophys Acta.* 1999, 1411: 334-50.

Eiserich J.P., Estevez A.G., Bamberg T.V., Ye Y.Z., Chumley P.H., Beckman J.S., Freeman B.A. Microtubule dysfunction by posttranslational nitrotyrosination of alpha-tubulin: a nitric oxide-dependent mechanism of cellular injury. *Proc. Natl. Acad. Sci. U.S.A.* 1999, 96: 6365-70.

Epe B., Ballmaier D., Roussyn I., Briviba K., Sies H. DNA damage by peroxynitrite characterized with DNA repair enzymes. *Nucleic Acids Res.* 1996, 24: 4105-10.

Fan T.Y., Morrison J., Rounbehler D.P., Ross R., Fine D.H., Miles W., and Sen N.P. N-Nitrosodiethanolamine in synthetic cutting fluids: a part-per-hundred impurity. *Science.* 1977, 196 : 70-71.

Fan T.Y., Morrison J., Rounbehler D.P., Ross R., Fine D.H., Miles W., Sen N.P., Freedman J.E., Loscalzo J., Benoit S.E., Valeri C.R., Barnard M.R., Michelson A.D. Decreased platelet inhibition by nitric oxide in two brothers with a history of arterial thrombosis. *J. Clin. Invest.* 1996, 97: 979-987.

Feelisch M. Biotransformation to nitric oxide of organic nitrates in comparison to other nitrovasodilators. *Eur Heart J.* 1993, 14: 123-32.

Feldman P.L., Griffith O.W., Stuehr D.J. The surprising life of nitric oxide . *Chem. Eng. News.* 1993, 26-38.

Feelisch M., and Stamler J.S. in Methods in Nitric Oxide Research, eds. Feelisch, M. & Stamler J. S. (Wiley, U.K.)1996, pp. 455-478.

Flohe, L. Glutathione peroxidase. *Basic-Life-Sci.* 1988, 49663-8

Frostell C., Fratacci M.D., Wain J.C., Jones R., Zapol W.M. Inhaled nitric oxide. A selective pulmonary vasodilator reversing hypoxic pulmonary vasoconstriction. *Circulation.* 1991, 83: 2038-47.

Gardner P.R., Costantino G., Szabo C., Salzman A.L. Nitric oxide sensitivity of the aconitases. *J. Biol. Chem.* 1997, 272: 25071-6.

Garthwaite J., and Boulton C.L., Niric oxide signaling in central nervous system. *Annu.Rev.Physiol.* 1995, 57: 683-706.

Gaston B. Nitric oxide and thiol groups. Biochim Biophys Acta. 1999, 1411: 323-33.

Gaston B., Drazen J.M., Jansen A., Sugarbaker D.A., Loscalzo J., Richards W., Stamler J.S. Relaxation of human bronchial smooth muscle by S-nitrosothiols *in vitro*. *J. Pharmacol. Exp. Ther*. 1994, 268: 978-84.

Gaston B., Reilly J., Drazen J.M., Fackler J., Ramdev P., Arnelle D., Mullins M.E., Sugarbaker D.J., Chee C., Singel D.J., *et. al.* Endogenous nitrogen oxides and bronchodilator S-nitrosothiols in human airways. *Proc. Natl. Acad. Sci. U.S.A.* 1993, 90: 10957-61.

Geller D.A., and Billiar T.R. Molecular biology of nitric oxide synthases. *Cancer Metastasis Rev.* 1998, 17: 7-23.

Grisham M.B., Ware K., Gilleland H.E., Jr., Gilleland L.B., Abell C.L., Yamada T. Neutrophil-mediated nitrosamine formation: role of nitric oxide in rats. *Gastroenterology*. 1992, 103: 1260-6.

Hausladen A., Privalle C.T., Keng T., DeAngelo J., and Stamler J.S. Nitrosative stress: activation of the transcription factor OxyR. *Cell.* 1996, 86: 719-729.

Hayes J.D., and Pulford D.J. The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. *Crit. Rev. Biochem. Mol. Biol.* 1995, 30: 445-600.

Heales S.J., Bolanos J.P., Stewart V.C., Brookes P.S., Land J.M., Clark J.B., Nitric oxide, mitochondria and neurological disease. *Biochim. Biophys. Acta.* 1999, 1410: 215-28.

Heinecke J.W. Mechanisms of oxidative damage of low density lipoprotein in human atherosclerosis. *Curr. Opin. Lipidol.* 1997, 8: 268-74.

Heinecke J.W., Rosen H., Suzuki L.A., Chait. The role of sulfur-containing amino acids in superoxide production and modification of low density lipoprotein by arterial smooth muscle cells. *J. Biol. Chem.*1987, 262: 10098-10103.

Ignarro L.J. Biosynthesis and metabolism of endothelium-derived nitric oxide. *Annu. Rev. Pharmacol. Toxicol.* 1990, 30: 535-60.

Ignarro L.J., Edwards J.C., Gruetter D.Y., Barry B.K., Gruetter C.A. Possible involvement of S-nitrosothiols in the activation of guanylate cyclase by nitroso compounds. *FEBS Lett.* 1980, 110: 275-8.

Ignarro L.J., Lippton H., Edwards J.C., Baricos W.H., Hyman A.L., Kadowitz P.J., Gruetter C.A. Mechanism of vascular smooth muscle relaxation by organic nitrates, nitrites, nitroprusside and nitric oxide: evidence for the involvement of S-nitrosothiols as active intermediates. *J. Pharmacol. Exp. Ther.* 1981, 218: 739-49.

Incze K., Farkas J., Mihalyi V., Zukal E. Antibacterial effect of cysteine-nitrosothiol and possible percursors thereof. *Appl. Microbiol.* 1974, 27: 202-5.

Ischiropoulos H. Biological tyrosine nitration: a pathophysiological function of nitric oxide and reactive oxygen species. *Arch. Biochem. Biophys.* 1998, 356: 1-11.

Ischiropoulos H., and Al-Mehdi A.B. Peroxynitrite-mediated oxidative protein modifications. *FEBS Lett.* 1995, 364: 279-82.

Ischiropoulos H., Zhu L., Beckman J.S. Peroxynitrite formation from activated rat alveolar macrophage. *Arch. Biochem. Biophys.* 1992, 298: 446-51.

Iyengar R., Stuehr D.J., Marletta M.A. Macrophage synthesis of nitrite, nitrate, and N-nitrosamines: precursors and role of the respiratory burst. *Proc. Natl. Acad. Sci. U.S.A.* 1987, 84: 6369-73.

Jakobsson P.J., Morgenstern R., Mancini J., Ford-Hutchinson A., Persson B. Common structural features of MAPEG - a widespread superfamily of membrane associated proteins with highly divergent functions in eicosanoid and glutathione metabolism. *Protein Sci.* 1999b, 3: 689-692.

Jakobsson P.J., Thoren S., Morgenstern R., Samuelsson B**.,** Identification of human prostaglandin E synthase: a microsomal, glutathione-dependent, inducible enzyme, constituting a potential novel drug target. *Proc. Natl. Acad. Sci. U.S.A.* 1999a, 96: 7220-5.

Jourd'heuil D., Hallen K., Feelisch M., Grisham M.B., Dynamic state of S-nitrosothiols in human plasma and whole blood. *Free Radic. Biol. Med.* 2000, 28: 409-17.

Kamisaka K., Habig W.H., Ketley J.N., Arias I.M., Jakoby W.B., Multiple forms of human glutathione S-transferase and their affinity for bilirubin. *Eur. J. Biochem.* 1975, 60:153-161.

Kang S.S., Wong P.W.K., Bock H.G., Horwitz A., Grix A. Intermediate homocysteinenemia resulting from compound heterozygosity of methylenetetrahydrofolate reductase mutations. *Am. J. Hum. Genet.* 1991, 48: 546-551.

Kang S.S. Wong P.W.K., Malinow M.R. Hyperhomocyst(e)inenemia as a risk factor for occlusive vascular disease. *Ann. Rev. Nutr.* 1992: 12, 259-268.

Kashiba M., Kasahara E., Chien K.C., and Inoue M.. Fate and vascular action of S-Nitrosoglutathione and related compounds in the circulation. *Arch. Biochem. Biophys*. 1999, 363: 213-218.

Ketterer B., Meyer D.J., Clark A.G. In Glutathione Conjugation: Mechanisms and Biological Significance (Sies H. and Ketterer B., eds.) Academic Press, London, 1988, pp. 74-126.

Kluge I., Gutteck-Amsler U., Zollinger M., Do KQ. S-Nitrosoglutathione in rat cerebellum: Identification and quantification by liquid chromatography-mass spectrometry. *J. Neurochem.* 1997, 69: 2599-607.

Koppenol W.H. The basic chemistry of nitrogen monoxide and peroxynitrite. *Free Radic. Biol. Med.* 1998, 25: 385-91.

Koppenol W.H., Kissner R., Beckman. J.S. Syntheses of peroxynitrite : To go with flow or on solid grounds? *Methods Enzymol.* 1996, 269, 296-302.

Koppenol W.H., Moreno J.J., Pryor W.A., Ischiropoulos H., Beckman J.S. Peroxynitrite, a cloaked oxidant formed by nitric oxide and superoxide. *Chem. Res. Toxicol.* 1992, 5(6):834-42.

Kosaka H., Wishnok J.S., Miwa M., Leaf C.D., Tannenbaum S.R**.** Nitrosation by stimulated macrophages: Inhibitors, enhancers and substrates. *Carcinogenesis*. 1989, 10: 563-6.

Kyhse-Andersen J. Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. *J. Biochem. Biophys. Methods.* 1984, 10: 203-209.

Laemmli U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature*.1970, 680-685.

Laskin D.L., Heck D.E., Gardner C.R., Feder L.S., Laskin J.D. Distinct patterns of nitric oxide production in hepatic macrophages and endothelial cells following acute exposure of rats to endotoxin. *J. Leukoc. Biol.* 1994, 56: 751-8.

Lewis R.S., Tamir S., Tannenbaum S.R., Deen W.M. Kinetic analysis of the fate of nitric oxide synthesized by macrophages *in vitro*. *J. Biol. Chem.* 1995, 270: 29350-5.

Lijinsky W., Reuber M.D., Manning W.B. Potent carcinogenicity of nitrosodiethanolamine in rats. Nature. 1980, 288: 589-90.

Lipton S.A., Choi Y.B., Pan Z.H., Lei S.Z., Ken H.S.V., Sucher N.J., Loscalzo J., Single D.J., and Stamler J.S. A redox–based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso–compounds. *Nature*.1993, 364: 626-632.

Lipton S.A, Kim W.K., Choi Y.B., Kumar S., D'Emilia D.M., Rayudu P.V., Arnelle D.R., Stamler J.S. Neurotoxicity associated with dual actions of homocysteine at the N-methyl-D-aspartate receptor. *Proc. Natl. Acad. Sci. U.S.A.* 1997 94: 5923-8.

Lipton S.A., Singel D.J., Stamler J.S. Neuroprotective and neurodestructive effects of nitric oxide and redox congeners. *Ann. N. Y. Acad. Sci.* 1994, 738: 382-7.

Liu R.H., and Hotchkiss J.H. Potential genotoxicity of chronically elevated nitric oxide: a review. *Mutat. Res*. 1995, 339: 73-89.

Liu R.H., Jacob J.R., Tennant B.C., Hotchkiss J.H. Nitrite and nitrosamine synthesis by hepatocytes isolated from normal woodchucks (Marmota monax) and woodchucks chronically infected with woodchuck hepatitis virus. *Cancer Res.* 1992, 52: 4139-43.

Loeppky R.N. The mechanism of bioactivation of N-nitrosodiethanolamine. *Drug Metab. Rev.* 1999, 31: 175-93.

Loeppky R.N., Lee M.P., Mueller S. DNA modification by -nitrosamino aldehydes. In The Chemistry of DNA Adducts (Hemminki, K., and Bartsch, H., Eds.), International Agency for Research on Cancer, Lyon, France. 1993, pp 429-432

Loeppky R.N., Tomasik W., Denkel E., and Eisenbrand G. Nitrosaminoaldehydes: highly reactive metabolites. *IARC Sci. Publ.* 1987, 84: 94-99.

Loeppky RN., Tomasik W., and Kerrick B.E. Nitroso transfer from -nitrosamino aldehydes: Implications for carcinogenesis. *Carcinogenesis*. 1987,8, 941-946.

Maeda H., Okamoto T., Akaike T. Human matrix metalloprotease activation by insults of bacterial infection involving proteases and free radicals. *Biol Chem.* 1998, 379: 193-200.

MacMillan-Crow L.A., Crow J.P., Kerby J.D., Beckman J.S., Thompson J.A. Nitration and inactivation of manganese superoxide dismutase in chronic rejection of human renal allografts. *Proc. Natl. Acad. Sci. U.S.A.* 1996, 93: 11853-8

Malinow M.R. Homocyst(e)ine and arterial occulisive disease. *J. Int. Med.* 1994, 236: 603-617.

Malinow M.R., Kang S.S., Taylor L.M., Wong P.W.K., Inahara T., Mukerjee D., Sexton G., and Upson B. Prevalence of hyperhomocyst(e)inenemia in patients with peripheral arterial occlusive disease. *Circulation.* 1989, 79: 1180-1188.

Mannervik B., and Widersten M. in Advances in Drug Metabolism in Man (Pacifici, G. M., and Fracchia, G. N., Eds.) European Commission, Luxembourg. 1995, pp 407-460.

Mannervik B., Danielson U.H. Glutathione transferases-structure and catalytic activity. *CRC Crit Rev Biochem.* 1988, 23: 283-337.

Mansoor M.A., Bergmark C., Svardal A.M., Lonning P.E., Ueland P.M. Redox status and protein binding of plasma homocyst(e)ine and other aminothiols in patients with early-onset peripheral vascular disease. *Arterio. Thromb. Vasc. Biol.* 1995, 15:232-240.

Marletta M.A, Hurshman A.R., Rusche K.M. Catalysis by nitric oxide synthase. *Curr. Opin. Chem. Biol.* 1998: 656-63.

Masuda M., Mower H.F., Pignatelli B., Celan M., Friesen M.D., Nishino H., and Ohshima, H. Formation of N-nitrosamines and N-nitramines by the reaction of secondary amines with peroxynitrite and other reactive nitrogen species: comparison with nitrotyrosine formation. *Chem. Res. Toxicol.* 2000, 13: 301-308.

Mathews W.R., and Kerr S.W**.** Biological activity of S-nitrosothiols: the role of nitric oxide. *J. Pharmacol. Exp. Ther.* 1993, 267: 1529-37.

Matsudaira P. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J. Biol. Chem.* 1987, 262: 10035-10038

McAndrew J, Patel R.P., Jo H., Cornwell T., Lincoln T., Moellering D., White C.R., Matalon S., Darley-Usmar V. The interplay of nitric oxide and peroxynitrite with signal transduction pathways: implications for disease. *Semin Perinatol.* 1997, 21: 351-66.

McAninly J., Williams D.LH., Askew S.C*. et. al.* Metal ion catalysis in nitrosothiol (RSNO) decomposition. *J. Chem. Soc. Chem. Commun.* 1758-9.

McCully K.S. Vascular pathology of homocysteinenemia: implications for the pathogenesis of arteriosclerosis. *Am. J. Pathol.* 1969, 56: 111-128.

Miwa M. Stuehr DJ. Marletta MA. Wishnok JS. Tannenbaum SR. Nitrosation of amines by stimulated macrophages. *Carcinogenesis.* 1987, 8(7):955-8.

Moncada S., and Higgs A. The L-arginine-nitric oxide pathway. *N Engl J Med.* 1993, 329: 2002-12.

Moncada S., Palmer R.M., Higgs E.A. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev.* 1991, 43: 109-42.

Morgenstern R., DePierre J.W., Ernster L. Activation of microsomal glutathione *S*-transferase activity by sulfhydryl reagents. *Biochem Biophys Res Commun.* 1979, 87: 657-63.

Morgenstern R., DePierre J.W., Jörnvall H. Microsomal glutathione transferase: Primary structure. *J. Biol. Chem.* 1985, 260: 13976-13983.

Morgenstern R., Lundquist G., Jörnvall H., DePierre J.W. Activation of rat liver microsomal glutathione transferase by limited proteolysis. *Biochem J.* 1989, 260: 577-82.

Morgenstern R., Wallin H., DePierre J.W. in Glutathione S-transferases and Carcinogenesis (Mantle, T. J., Pickett, C. B., and Hayes, J. D., Eds.) Taylor and Francis, London. 1987, pp 29-38.

Morris S.L., and Walsh R.C., Hansen J.N. Identification and characterization of some bacterial membrane sulfhydryl groups which are targets of bacteriostatic and antibiotic action. *J. Biol. Chem.* 1993, 259: 13590-4.

Morrison H.I., Schauble D., Desmeules M., Wigle D.T. Serum folate and risk of fatal coronary heart disease. *J. Am. Med. Assoc.*1996, 275: 1893-1896.

Morrow P.E., Toxicological data on NOx: an overview. *J Toxicol Environ Health.* 1984, 13: 205-27.

Mudd S.H., and Lew H.L. Plasma homocyst(e)ine or homocysteine ? *N. Engl. J. Med.* 1995, 274: 1049-1057.

Mudd S.H., and Levy H.L. Disorders in transsulfuration. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. The metabolic basis of inherited disease, 6th ed. McGraw-Hill: **New York, 1989, 693-734.**

Murphy M.P., Packer M.A., Scarlett J.L., Martin S.W. Peroxynitrite: a biologically significant oxidant. *Gen Pharmacol.* 1998, 31: 179-86.

Nathan C. Nitric oxide as a secretory product of mammalian cells. *FASEB J.* 1992, 6: 3051-64.

Nathan C. Inducible nitric oxide synthase: what difference does it make? *J Clin Invest.* 1997, 100: 2417-23.

Nathan C., and Xie Q.W. Nitric oxide synthases: roles, tolls, and controls. *Cell.* 1994, 78: 915-8.

Nikitovic D., and Holmgren A.. S-nitrosoglutathione is cleaved by the thioredoxin system with liberation of glutathione and redox regulating nitric oxide. *J. Biol. Chem.* 1996, 271:19180-19185.

Noack E., and Feelisch M. Molecular mechanisms of nitrovasodilator bioactivation. Basic Res Cardiol. 1991, 86: 37-50.

Norman V., and Keith C.H. Nitrogen oxides in tobacco somke. *Nature.* 1965, 205: 915-916.

Oh-hashi K., Maruyama W., Yi H., Takahashi T., Naoi M., Isobe K. Mitogen-activated protein kinase pathway mediates peroxynitrite-induced apoptosis in human dopaminergic neuroblastoma SH-SY5Y cells. *Biochem Biophys Res Commun.* 1999, 263: 504-9.

Ohshima H., and Bartsch H. Chronic infections and inflammatory processes as cancer risk factors: possible role of nitric oxide in carcinogenesis. *Mutat Res.* 1994, 305: 253-64.

Ohshima H., Gilibert I., Bianchini F. Induction of DNA strand breakage and base oxidation by nitroxyl anion through hydroxyl radical production. *Free Radic Biol Med.* 1999, (9-10): 1305-13.

Ohshima H., Tsuda M., Adachi H., Ogura T., Sugimura T., Esumi H. L-arginine-dependent formation of N-nitrosamines by the cytosol of macrophages activated with lipopolysaccharide and interferon-gamma. *Carcinogenesis.* 1991, 12: 1217-20.

Oakley B.R , Kirsch D.R., Morris N.R. A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. *Anal. Chem.* 1980, 105:361-363

Patel R.P., McAndrews J., Sellak H., White C.R., Jo H., Freeman V.M., Darley-Usmar V.M. Biological aspects of reactive nitrogen species. *Biochim. Biophys. Acta.* 1999, 1411: 385-400.

Pietraforte D., Mallozzi C., Scorza G., Minetti M. Role of thiols in the targeting of S-nitroso thiols to red blood cells. *Biochemistry*. 1995, 34:7177-85

Preussmann R., Habs M., Habs H., Schmahl D. Carcinogenicity of N-nitrosodiethanolamine in rats at five different dose levels. *Cancer Res.* 1982, 42: 5167-71.

Pryor W.A., and Squadrito G.L. The chemistry of peroxynitrite: a product from the reaction of nitric oxide with superoxide. *Am J Physiol.* 1995, 268: L699-722.

Radomski M.W., Rees D.D., Durta A., Moncada S. S-Nitrosoglutathione inhibits platelet activation *in vitro* and *in vivo*. *Br. J. Pharmacol.* 1993, 107: 745-9.

Roberts E.S., Lin H.l., Crowley J.R., Vuletich J.L., Osawa Y., Hollenberg P.F. Peroxynitrite-mediated nitration of tyrosine and inactivation of the catalytic activity of cytochrome P450 2B1. *Chem Res Toxicol.* 1998, 11: 1067-74.

Robinson K., Mayer E.L., Miller D.P., Green R., Vanlente F., Gupta A., Kottkemarchant K., Savon S.R., Selhub J., Nissen S.E., Kunter M., Topol E.J., Jacobsen D.W. Hyperhomocyst(e)inenemia and low pyridoxal phosphate: common and independent reversible risk factors for coronary artery disease. *Circulation.* 1995, 92: 2825-2830.

Salvemini D. Regulation of cyclooxygenase enzymes by nitric oxide. *Cell Mol Life Sci.* 1997, 53: 576-82.

Sampson J.B., Ye Y., Rosen H., Beckman J.S. Myeloperoxidase and horseradish peroxidase catalyze tyrosine nitration in proteins from nitrite and hydrogen peroxide. *Arch Biochem Biophys.* 1998, 356(2):207-13.

Scharfstein J.S., Keany J., Stamler J.S., et. al. Low molecular weight thiols transfer nitric oxide from an endogenous plasma reservoir to vascular smooth muscle. 1993, *Clin. Res.* 41:232A.

Schieke S.M., Briviba K., Klotz L.O., Sies H. Activation pattern of mitogen-activated protein kinases elicited by peroxynitrite: attenuation by selenite supplementation. *FEBS Lett.* 1999, 448: 301-3.

Schmidt H.H., and Walter U. NO at work. *Cell.* 1994, 78: 919-25.

Scorza G., Pietraforte D., and Minetti M. Role of ascorbate and protein thiols in the release of nitric oxide from S-nitrosoalbumin and S-nitrosoglutathione in human plasma. *Free Radical Biol.Med.* 1997, 22: 633-642.

Selhub J., Jacques P.F., Bostom A.G., D,Agostino R.B., Wilson P.W.F., Belanger A.J., O,Leary D.H., Wolf P.A., Rush D., Schaefer E.J., Rosenberg I.H. Relationship between plasma homocysteine, vitamin status and extracranial carotid-artery stenosis in the Framingham study population. *J. Nutr.* 1996, 126: S1258-S1265.

Selhub J., Jacques P.F., Wilson W., Rush D., Rosenberg I.H., Vitamin status and intake as primary determinants of homocysteinemia in an elderly population. *J. Am. Med. Assoc.*1993, 270: 2693-2698.

Sies H. Oxidative stress: from basic research to clinical application. *Am J Med.* 1991, 91:31S-38S.

Sies H. Glutathione and its role in cellular functions. *Free Radic Biol Med.* 1999, 27: 916-21

Singh R.J., Hogg N., Joseph J., Kalyanaraman B. Mechanism of nitric oxide release from S-nitrosothiols. *J Biol Chem.* 1996, 271: 18596-603.

Singh S.P., Wishnok J.S., Keshive M., Deen W.M., Tannenbaum S.R. The chemistry of the S-nitrosoglutathione/glutathione system. *Proc Natl Acad Sci U.S.A.* 1996, 93: 14428-33.

Souza J.M., and Radi R. Glyceraldehyde-3-phosphate dehydrogenase inactivation by peroxynitrite. *Arch Biochem Biophys.* 1998, 360: 187-94.

Stachowiak O., Dolder M., Wallimann T., Richter C. Mitochondrial creatine kinase is a prime target of peroxynitrite-induced modification and inactivation. *J Biol Chem.* 1998, 273: 16694-9.

Stamler J.S. S-nitrosothiols and the bioregulatory actions of nitrogen oxides through reactions with thiol groups. *Curr Top Microbiol Immunol.* 1995, 196: 19-36.

Stamler J.S., Jaraki O., Osborne J., Simon D.I., Keaney J., Vita J., Singel D., Valeri C.R., Loscalzo J. Nitric oxide circulates in mammalian plasma primarily as an S-nitroso adduct of serum albumin. *Proc Natl Acad Sci U.S.A.* 1992a, 89: 7674-7.

Stamler J.S., Loh E., Roddy M.A., Currie K.E., Creager M.A. Nitric oxide regulates basal systemic and pulmonary vascular resistance in healthy humans. *Circulation.* 1994, 89: 2035-40.

Stamler J.S., Osborne J.A., Jaraki O., Rabbani L.E., Mullins M.E., Singel D.J., Loscalzo J. Adverse vascular effects of homocysteine are modulated by endothelium-derived relaxing factor and related oxides of nitrogen. *J. Clin. Invest.* 1993, 91: 308-318.

Stamler J.S., Singel D.J., Loscalzo J. Biochemistry of nitric oxide and its redox-activated forms. Science. 1992b, 258: 1898-902.

Stampfer M.J., Malinow M.R., Willett W.C., Newcomer L.M., Upson B., Ullmann D., Tishler P.V., Hennekens C.H. A prospective study of plasma homocyst(e)ine and risk of myocardial infarction in US physicians. *JAMA.* 1992, 268: 877-81.

Starkebaum G., and Harlan L.A. Endothelial cell injury due to copper-catalyzed hydrogen peroxide generation from homocysteine. J. Clin. Invest. 1986, 77: 1370-1376.

Stuehr D.J. Structure-function aspects in the nitric oxide synthases. *Annu Rev Pharmacol Toxicol.* 1997, 37: 339-59

Stuehr D.J. Mammalian nitric oxide synthases. *Biochim. Biophys. Acta.* 1999, 1411: 217-330.

Szabados E., Fischer G.M., Gallyas F., Jr., Kispal G., Sumegi B. Enhanced ADP-ribosylation and its diminution by lipoamide after ischemia-reperfusion in perfused rat heart. *Free Radic Biol Med.* 1999, 27: 1103-13.

Tamir S. and Tannenbaum SR. The role of nitric oxide (NO.) in the carcinogenic process. *Biochim Biophys Acta.* 1996,1288: F31-6.

Tannenbaum S.R. Sinskey AJ. Weisman M. Bishop W. Nitrite in human saliva. Its possible relationship to nitrosamine formation. J Natl Cancer Inst. 1974, 53: 79-84.

Tasker H.S., and Jones H.Q. The action of mercaptans on acid chlorides: The acid chlorides of phosphorus, sulfur and nitrogen. *J. Chem. Soc.* 1995, Part II, 1910.

Tew K.D. Glutathione-associated enzymes in anticancer drug resistance. *Cancer Res.* 1994, 54: 4313-20.

Ubbink J.B., Vandermerwe A., Delport R., Allen R.H., Stabler S.P., Riezler R., Vermaak W.J.H. The effect of a subnormal vitamin B-6 status on homocysteine metabolism. *J. Clin. Invest.* 1996, 98: 177-184.

Ubbink J.B., Vermaak W.J.H., Vandermerwe A., Becker P.J., Delport R., Potgieter H.C. Vitamin requirements for the treatment of hyperhomocysteinemia in humans. *J. Nutr.* 1994, 124: 1927-1933.

Upchurch G.R., Welch G.N., Freedman J.E., Loscalzo J. homocyst(e)ine attenuates endothelial glutathione peroxidase and thereby potentiates peroxide-mediated cell injury. *Circulation.* 1995, 92: 1-228.

Upchurch G.R., Welch G.N., Loscalzo J. The vascular biology of S-nitrosothiols, nitrosated derivatives of thiols. *Vasc Med.* 1996, 1: 25-33.

Upchurch G.R., Jr., Welch G.N., Loscalzo J. Homocysteine, EDRF, and endothelial function. *J Nutr.* 1996, 126: 1290S-4S.

Upchurch G.R., Jr., Welch G.N., Fabian A.J., Freedman J.E., Johnson J.L., Keaney J.F., Loscalzo J. Homocyst(e)ine decreases bioavailable nitric oxide by a mechanism involving glutathione peroxidase. *J Biol Chem.* 1997, 272: 17012-7.

Upchurch G.R., Jr., Welch G.N., Fabian A.J., Pigazzi A., Keaney J.F., Loscalzo J. Stimulation of endothelial nitric oxide production by homocyst(e)ine. *Atherosclerosis.* 1997, 132: 177-85.

van der Vliet A., Chr.´t Hoen P. A., Wong P. S., Bast A., and Cross C.E. Formation of S-nitrosothiols via direct nucleophilic nitrosation of thiols by peroxynitrite with elimination of hydrogen peroxide. *J. Biol. Chem.* 1998, 273: 30255-30262.

Wall R.T., Harlan J.M., Harker L.A., Striker G.E. Homocyst(e)ine-induced endothelial cell injury in vitro: a model for the study of vascular injury. *Thromb. Res.* 1980, 18: 113-121.

Wang K., Wen Z., Zhang W., Xian M., Cheng J.P., Wang P.G. Equilibrium and kinetics studies of transnitrosation between S-nitrosothiols and thiols. *Bioorg Med Chem Lett.* 2001,11: 433-6.

Weinander R., Ekstrom L., Andersson C., Raza H., Bergman T., Morgenstern R., Structural and functional aspects of rat microsomal glutathione transferase. The roles of cysteine 49, arginine 107, lysine 67, histidine, and tyrosine residues. *J. Biol. Chem.* 1997, 272:8871-7.

Welch G.N., Upchurch G.R., Jr., Loscalzo J. Homocysteine, oxidative stress, and vascular disease. Hosp Pract (Off Ed). 1997, 32(6):81-2, 85, 88-92.

Wink D.A., and Laval J., The Fpg protein, a DNA repair enzyme, is inhibited by the biomediator nitric oxide in vitro and in vivo. *Carcinogenesis.* 1994, 15: 2125-9.

Xian M., Wang Q.M., Chen X., Wang K., Wang P.G. S-nitrosothiols as novel, reversible inhibitors of human rhinovirus 3C protease. *Bioorg Med Chem Lett.* 2000, 10: 2097-100.

Zhu L., Gunn C., Beckman J.S. Bactericidal activity of peroxynitrite. *Arch Biochem Biophys.* 1992, 298: 452-7.

Zou M., Martin C., Ullrich V. Tyrosine nitration as a mechanism of selective inactivation of prostacyclin synthase by peroxynitrite. *Biol Chem.* 1997, 378: 707-13.

7. Acknowledgements

The present thesis has been performed at the Institute für Physiologische Chemie I, Heinrich-Heine-Universität, Düsseldorf.

I wish to express my deep gratitude to everyone who has contributed to this thesis.

I especially want to thank:

My supervisor Prof. Dr. Dr. h. c. Helmut Sies for his generosity in sharing with me his broad knowledge, his unfailing optimism and also for his continuous encouragement during my “ups and downs”.

My deep thanks also to Prof. Dr. Wunderlich for sharing the responsibility of this work and together with Prof. Dr. Martin for their understanding and support.

My deep thanks also to Prof. Dr. Wilhelm Stahl for his continuous encouragement to finish this work, either during experimental work or through preparing this thesis and for guidance to select this study.

I extend my thanks to Dr. Akerboom for help and guidance at the beginning of my study especially during the GST studying.

My thanks are also directed to Peter Graf for his help on HPLC and valuable technical advice, Heide Krahl for help in organisation.

For the good working atmosphere I would like to thank my past and present colleagues of Prof. Dr. Stahl working group Dr. Cristina Polidori, Niloofar Ale-Agha, Olivier Aust, Alejandro Betancor, Dr. Lianfu Zhang,.

Marlies Scholtes is gratefully acknowledged for her excellent secretarial assistance and for always being there with a helping hand.

I also want to thank everybody (past and present) of the Institüt für Physiologische Chemie (Dr. Wagner, Dr. Claudio Jösch, Darius Buchczyk, Peter Schröder, and

Gisela Michael) who in one way or another have given support and help during all these years in the institute.

I would also like to thank Prof Dr. Hunitai who encouraged me to contact and find a PhD position with Prof. Dr. Dr. h. c. Helmut Sies.

Finally for the financial and psychological support throughout these investigations I would like to thank the foundation of the Katholische Akademische Ausländer Dienst (KAAD). Especially Mr. Hans Landsberg for his continuous help and support, Susana Körper (Katholische Hochschulgemeinde) for her help and Dr. Farah Al-Naser who recommended me for a scholarship of the KAAD, Germany.

My thanks and love go to my family in Jordan who has continuously supported and encouraged me in this work.

Curriculum Vitae

Name	: Ahmed Hussein Al-Mustafa
E-mail	: aahmed65@yahoo.com
Born	: 21.01. 1965, Rassoun-Ajloun, Jordan
Nationality	: Jordanien
Sex	: Male
Marital status	: Married

Education:

University:

April.98-Present	PhD. Degree in Physiological Chemistry I Dept., Heinrich-Heine-University, Düsseldorf, Germany. Thesis subject: STABILITY AND TRANSNITROSATION EFFICACY OF S-NITROSOTHIOLS IN BIOLOGICAL MODEL SYSTEMS.
Feb. 90-Jun. 93	M.Sc. degree in Biology, Yarmouk-Uni. Irbid-Jordan.
Sep.83-Jun.87	B.Sc. degree in Nutrition, Yarmouk-Uni. Irbid-Jordan.
July 83	General Secondary Certificate (Scientific Stream)

Professional:

Sep.97-April.98	Intensive German language Course, at Kreuzberg Institute-Bonn.

Work experience:

April.98-present	Research assistant, Institute of Physiological Chemistry I, Heinrich-Heine-University, Düsseldorf, Germany.
Feb.90-Oct.93	Research and teaching assistant, Biology Dept., Yarmouk-Uni., Irbid-Jordan.
Oct.93-Sep-97	Research and teaching assistant, Biology Dept., Mu'tah-Uni., Karak-Jordan.

Fellowship:

Sep.97-Oct.2001	Katholische Akademische Ausländer Dienst (KAAD)-Bonn, Germany.

Publications:

-**Ahmed H. Al-Mustafa**, Helmut Sies and Wilhelm Stahl. Sulfur-to-Nitrogen Transnitrosation: Transfer of Nitric Oxide from S-Nitroso Compounds to Diethanolamine and the Role of Intermediate Sulfur-to–Sulfur Transnitrosation. *Toxicology*. 2001,

-Lamya Al-Diwany, Khaled Tarawneh, and **Ahmed H. Al-Mustafa**. Detection of glutathione S-transferase in moderate halophilic bacteria from Al-Ghuir Agricultural station in Karak Governorate (South Jordan). *Mu'tah Lil-Buhuth wad-Dirasat.* 13,99-111