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ENZYMATIC TRANSFORMATION OF SOME VASODILATING SUBSTANCES TO NITRIC OXIDE

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My greatest thanks belong to my family for the support and understanding during my entire studies.

Declaration :

I affirm that I have worked out my thesis alone and have presented all references used.

Prague, April 18, 2006

CONTENT

1. LIST OF ABBREVIATIONS

ALDH	Aldehyde dehydrogenase
AO	Acetaldoxime
BSA	Bovine serum albumin
cGMP	Cyclic guanidine monophosphate
DC	3,3′-methylene-bis(4-hydroxycoumarine), dicoumarol
DMSO	Dimethylsulfoxide
DP	Diaphorase from *Clostridium kluyveri* (EC 1.8.1.4)
DPI	Diphenyleneiodonium chloride
EDRF	Endothelium-derived relaxing factor
eNOS	Endothelial nitric oxide synthase (EC 1.14.13.39)
EPR	Electronic paramagnetic resonance
ER	7-ethoxyresorufin, resorufin ethyl ether
FAD	Flavin adenine dinucleotide
FAL	Formaldoxime
FAM	Formamidoxime
FMN	Flavin mononucleotide
GTN	Glycerol trinitrate
HA	Hydroxylamine
Hb	Hemoglobin
iNOS	Inducible nitric oxide synthase
LAD	Lipoamide dehydrogenase from porcine heart (EC 1.8.1.4)
L-NAME	N_{ω}-Nitro-L-arginine methyl ester hydrochloride
MC	Miconazole nitrate salt
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide reduced form
NADPH	Nicotinamide adenine dinucleotide phosphate
NHA	N^{ω}-hydroxy-L-arginine
nNOS	Neuronal nitric oxide synthase
NOS	Nitric oxide synthase
NP	Nitroprusside
PA	Proadiphen hydrochloride
PH	1-Phenyl-3-pyrazolidinone, phenidone

PTIO	2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxdie
SEM	Standard error of the mean
sGC	Soluble guanylate cyclase
SNAP	S-nitroso-N-acetylpenicillamin / N-acetyl-3-(nitrosothio)-D-valine
SNOG	S-nitrosoglutathione (S-nitroso-γ-glutamylcysteinylglycine)
SOD	Superoxide dismutase
THB_4	Tetrahydrobiopterin
Tris	Tris[hydroxylmethyl]aminomethane; TRIZMA base
XO	Xanthine oxidoreductase

$O_2^{\cdot -}$	Superoxide anion
$OONO^-$	Peroxynitrite
NO	Nitric oxide (nitrogen II oxide)
$NO^{\cdot}$	Nitric oxide radical
NO^-	Nitroxyl anion
NO^+	Nitrosonium cation

2. THE AIMS OF THIS THESIS

Aims:

Contribution to our understanding of the mechanism(s) of enzymatic pathways involved in biotransformation of compounds with vasorelaxing action.

It has been shown in previous studies of our laboratory, that reactions of diaphorase from *Clostridium kluyveri* with glycerol trinitrate and formaldoxime (vasorelaxing substances) result in release of nitric oxide.

The aim of this thesis was to obtain precise kinetic parameters for reactions of diaphorase with glycerol trinitrate and formaldoxime. Furthermore we should try to obtain the same kinetic parameters for possible reaction of diaphorase with some other vasorelaxing substances (S-nitrosoglutathione, S-nitroso-N-acetylpenicilamine, nitroprusside, acetaldoxime and formamidoxime) and for the possible similar reaction of dihydrolipoamide dehydrogenase with the same vasorelaxing substances.

Inhibition studies are proposed to get a more precise insight into the mechanisms by which diaphorase and dihydrolipoamide dehydrogenase transform selected vasorelaxing compounds

3. INTRODUCTION

3.1. Nitric oxide

Nitric oxide (NO) was named "The molecule of the year" in 1992 by Science and in 1998, Furchgott, Ignarro and Murad were rewarded the Nobel Prize for their discoveries concerning nitric oxide as a signalling molecule in the cardiovascular system [1]. Due to the discovery of its endogenous biosynthesis and diversity of its biological actions, it is a molecule of extreme physiological, pharmacological and pathophysiological interest. Among its diverse functions, NO has been implicated in the relaxation of vascular smooth muscle, the inhibition of platelet aggregation, neurotransmission, immune regulation [2].

3.1.1. History of NO

The field of NO research started over a century ago by observation of the nitrate in urine from febrile patient [3] and after the discovery of glycerol trinitrate (GTN), later on, the vasorelaxating effect of nitrates was first appreciated as a treatment of angina pectoris [4]. In 1977 a stimulation of guanylate cyclase (GC) by various vasodilator compounds, like GTN and NO, was reported by F. Murad *et al.* His group even suggested that this effect could be associated with NO formation [5]. Three years later Furchgott and Zawadski described an endothelium-dependent relaxation of blood vessels upon precontraction with phenylephrine and suggest a release of a substance after activating muscarinic receptors on endothelial cells [6]. This substance was than called endothelium-derived relaxing factor (EDRF) [7]. The stimulation of vascular smooth muscle by EDRF increased cGMP levels [8,9]. The properties of EDRF and NO were thoroughly compared and in 1987, both Ignarro´s and Palmer´s group indeed reported that EDRF is NO [10, 11]. One year later the biosynthesis from L-arginine was discovered [12].

3.1.2. The chemical properties of NO

NO is a simple, diatomic molecule, with odd number of electrons, thus a free radical. NO is a colorless gas at room temperature and pressure. It has a limited solubility in water (2-3 $mmol.l^{-1}$) and possesses 6- to 8-fold higher solubility in nonpolar solvents [13] and lipid membranes compared to water. NO is a readily diffusible molecule with half-life estimated to be up to few seconds (in aqueous solution cca 4 s) [14]. And therefore it cannot be transported away from its source of synthesis. In addition, in physiological environment this radical can undergo numerous reactions with other radicals, and this often results in the formation of

additional reactive nitrogen oxide species: nitrosonium, NO^+ *[15] and nitroxyl anion,* NO^- [16]. The most biologically relevant reactions are primarily with O_2, with the superoxide anion $O_2^{\cdot-}$ and with the transition metals. The products of the reaction, NO_2^-, metal-NO adducts and peroxynitrite $OONO^-$ react further by nitrosylation of nucleophilic centers. Thus, NO can conceivably be either oxidized or reduced and the whole biological action as an effector and /or mediator is along with the molecules derived from it [17]. In aqueous solution or blood, the only stable endproduct is nitrite with small amounts of nitrates [18], but concentration of nitrate increase in presence of another oxidizing agent [19].

3.1.3. How does NO act?

How the molecule can travel through the body since it is very reactive? Classical extracellular messengers do not undergo any chemical reaction with the receptor, but NO can bring a covalent modification and even accumulation at the target protein. This modification is reversible. Stamler *et al.* [20] proposed that some thiol groups can be nitrosated and are thus capable of transporting NO to distal regions of the body. S-nitrosylation of proteins increased the stability and the half-life time up to several hours. Pawloski *et al.* [21] and Gross *et al.* [22] suggest that NO reacts with Fe^{2+} of hemoglobin (Hb), then it transfers to the SH group of the protein [23]. Interestingly, Demoncheaux *et al.* [24] have recently demonstrated that nitrites release small quantities of NO and so they believe that the vasodilator effect is due directly to nitrites. However, Froehlich et al. [25] argue that even though the quantities of NO released by nitrites are very small, such small quantities can react with thiols, whose activity as a vasodilator is much higher than the activity of free NO. The reactions with hem and nonhem iron [26], thiols [27] or amines [28] as well as with other various chemical substances are known. Adducts with amines, thiols and other nucleophiles can in turn slowly and spontaneously release nitric oxide by first-order kinetics. 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) or hemoglobin are widely used as a NO-scavenger. Oxyhemoglobin, a large molecule that cannot readily cross cell membranes and binds NO with 10^6-fold higher affinity than O_2, is often used in many preparations or as a spin–trap for EPR. The breakdown of NO that occurs following interaction with superoxide is prevented by superoxide dismutase (SOD).

3.2. Introduction to NO biosynthesis

Endogenous NO is produced almost exclusively by L-arginine catabolism to L-citrulline in reaction catalyzed by a family of nitric oxide synthases (NOS, EC 1.14.13.39) [29]. The NOS exists in mammalian systems in four isoforms, three of them share about 50% amino acid homology and are encoded by different genes localized in different chromosomes and have different tissue distribution. The three known isoforms are the neuronal nNOS (NOS I), the inducible iNOS (NOS II) and the endothelial constitutive isoform eNOS (NOS III) [30, 31]. Existence of the fourth, mitochondrial NOS, is still discussed (Ghafourifar and Cadenas, 2005) [32, 33]. The eNOS and nNOS isoforms are present within the cardiovascular system, require presence of Ca^{2+} and produce low levels of NO. The iNOS in contrast, produces the largest bulk of NO and is not regulated by Ca^{2+}. It is inducible at the gene level by various agents, such as proinflammatory cytokines, bacteria-derived molecules (e.g. lipopolysacharides), hypoxia, neuronal activity and foreign DNA or RNA [34, 35].

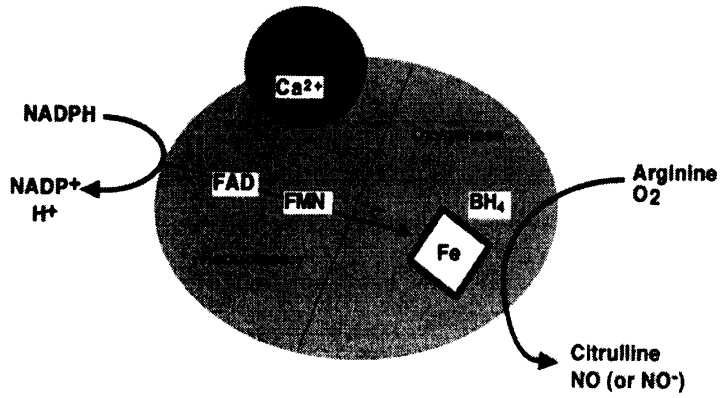

Fig.1 *Overall reaction catalyzed and cofactors of NOS* [35].
Electrons (e^-) are donated by NADPH to the reductase domain of the enzyme and proceed via FAD and FMN redox carriers to the oxygenase domain. There they interact with the haem iron and BH_4 at the active site to catalyze the reaction of oxygen with L-arginine, generating citrulline and NO as products. Electron flow through the reductase domain requires the presence of bound Ca^{2+}/CaM

NOS isoforms are dimers (two NOS monomers with two calmodulin binding motifs, Fig.1), with tightly bound tetrahydrobiopterin (BH_4), FAD, FMN, iron hem [36, 37, 38] and a zinc tetrathiolate center. All NOS catalyze basically identical reaction where from L-Arg, NADPH and oxygen a free radical NO, citrulline and NADP are produced. However, some authors suggest the possibility that the side product of NOSs is nitroxyl anion NO^- [39, 40]. NO

synthesis involves in the first step generating the enzyme-bound intermediate N^{ω}-hydroxy-L-arginine (NHA) [41], in the second step, NHA is oxidized to citrulline and NO (Fig. 2). Oxygen activation is carried out by the enzyme-bound heme, which derives electrons from NADPH [42]. Arginine can be than regenerated in urea cycle.

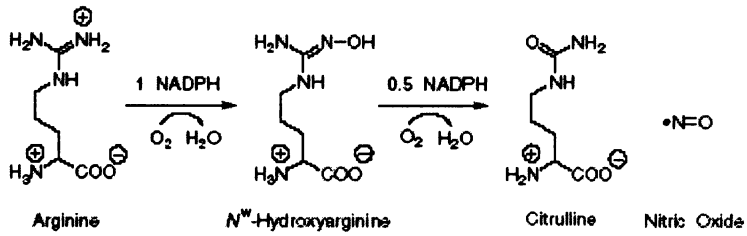

Fig.2. *NOS-catalyzed biosynthesis of NO*

3.2.1. Inhibition of NOS

There is a bewildering array of NOS inhibitors described in the literature and in the pharmacological tools. Of these the most widely used have been N-monomethyl-L-arginine, N-nitro-L-arginine and its methyl ester prodrug N-nitro-L-arginine methyl ester (L-NAME) [43]. A further group of inhibitors interacts with flavin (e.g. diphenyleneiodonium DPI) or with calmodulin (e.g. trifluorpiperazine)[44].

3.3. Regulatory function of NO - role of guanylyl cyclase (GC)

The mechanism of NO action is mediated by the activation of a soluble guanylyl cyclase (sGC). NO, freely crossing cell membrane reacts with the iron-containing heme moiety, and subsequently, the enzyme converts GTP in cGMP. Cyclic GMP, in turn, activates a cGMP-dependent protein kinase G that phosphorylates several intracellular molecules, which increase calcium reuptake in cells and inhibit calcium influx (see Fig.3). It leads to decrease of intracellular free Ca^{2+} and turn-off cell activation. The ring form of cGMP is subsequently cleaved by variety of phosphodiesterases [45]

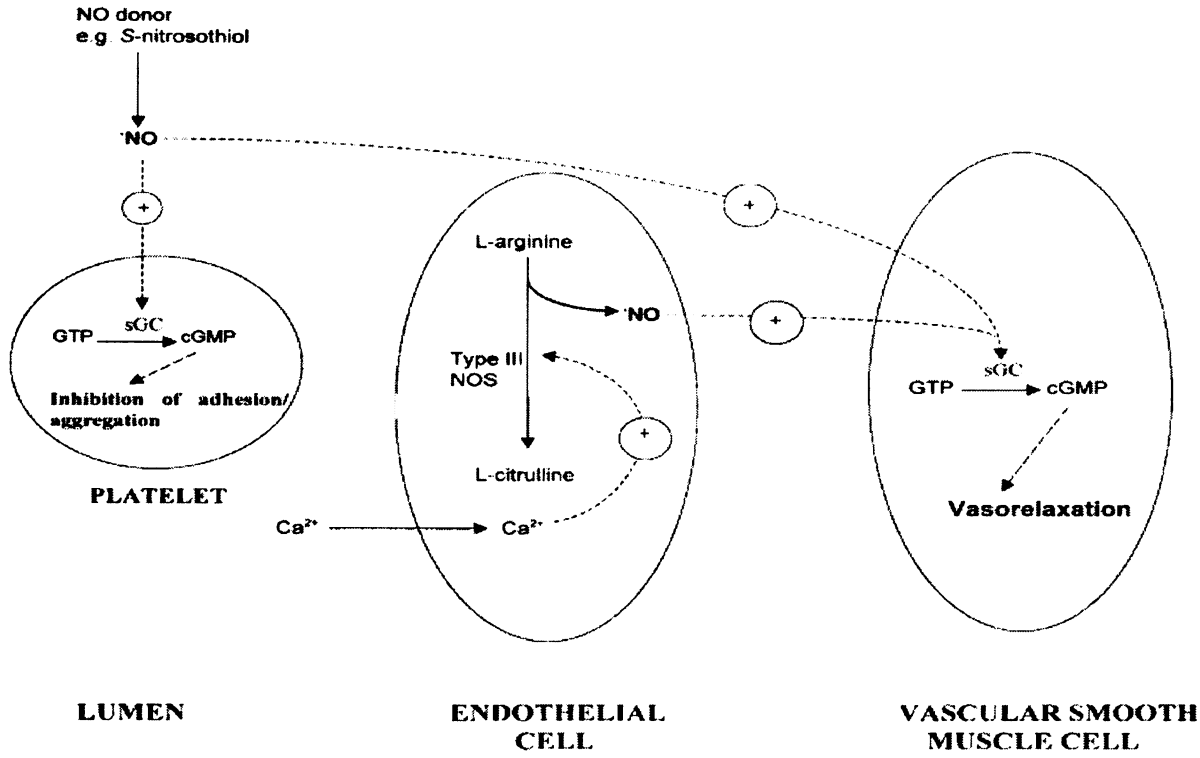

Fig. 3 *Vascular effects of NO, generated either by endothelial cells or by an exogenous source.*

3.4. Physiological function

NO has many effects both positive and deleterious. Its main effect is to dilate blood vessels and consequently to decrease arterial pressure. In the heart, it also plays an important role in keeping the vessels patent, prevention of platelet aggregation and regulating the force and rate of contraction.

Connected with the vasorelaxation is the rather famous effect of Viagra® on smooth muscle cells in masculine reproductive organs. The active ingredient in Viagra is a specific inhibitor of phosphodiesterase 5. The inhibition of the phosphodiesterase allows for the persistence and accumulation of cGMP and magnifying the NO action. The increased blood flow into the penis cavity may result in an erection. One of the curious side effects of Viagra is an effect on the visual discrimination of certain colors. It seems to be due to the reduced affinity of the drug for the phosphodiesterase 6, enzyme found in the retina. Viagra has an even more reduced affinity for phosphodiesterase 3, which is critical, since this isoenzyme is active in heart muscle cells [46].

3.4.1. Anticancer activity

NO can have influence on the growth of cancer. An overproduction of NO (by activated macrophages or exogenous) can enhance the radiation-induced apoptosis in cancer cells [47] or involving changes in mitochondrial permeability transition and release of cytochrome c from the mitochondria [48].

3.4.2. Cellular immune response

Overproduction of NO, mainly caused by inducible NOS, which is usually expressed by inflammatory phagocytic cells and other types of cells (e.g. epithelial and neuronal cells), has a defense function against bacteria, fungi and parasites.

3.4.3. Neurotransmitter and neuroendocrine function

NO acts on neuroendocrine and behavioural processes generally in one of three ways:

1. Indirectly, as NO derived from eNOS mediates blood flow.
2. Directly, as NO derived from nNOS affects neuronal functioning within the brain.
3. Hormonally, as NO derived from nNOS in endocrine glands or reproductive organs can regulate hormone release.

Much research has been carried out to understand NO-mediated regulation of neurotransmitter release and the relationship between NO and glucocorticoids. The release of the glucocorticoids is controlled by the brain and NO appears to play an important role in activating the hypothalamic-pituitary-adrenal axis [49].

3.4.4. Toxic action of NO

Cytotoxicity, oxidative stress, necrosis and apoptosis are associated with increased levels of NO, superoxide and their reaction product peroxinitrite. NO forms complexes with some metaloproteins and other enzymes, inhibiting key enzymes in DNA synthesis, mitochondrial respiration, iron metabolism, etc.

3.4.5. NO and virus infection

During a host's response against viral infection, NO is generated in excess, and contribute to viral pathogenesis by promoting oxidative stress and tissue injury. However, this common phenomenon enhances viral mutation, expands the quasispecies spectrum, and facilitates evolution of viruses [50]. NOS is induced in a variety of experimental virus infections in rats and mice, including Borna disease virus, herpes simplex virus or rabies virus. Expression of iNOS is also observed in human diseases caused by HIV-1 and hepatitis B virus [51]. The earliest host response to viral infections is non-specific, and involves induction of cytokines, especially tumor necrosis factor α and interferons. Cytokines are potent inducers of iNOS, which generates large amounts of nitric oxide [52].

Apoptosis
Oxidative stress
Antimicrobial effects
Increased vascular activity
Prevention of platelet aggregation
Inhibition of mitochondrial respiration
Vasodilatation of blood vessels
Decrease of arterial pressure
Anticancer activity
DNA damage
Cytotoxicity

Fig. 4 *Some of positive and deleterious effects of NO.*

3.5. NO donors

The replacement of defective endogenous NO by NO-donating molecules or NO biosynthesis stimulating compounds represents an important pharmacological tool. Besides supplementation of NO insufficiency in a pathological situation (cardiovascular disorders, nerve system diseases or inflammation), NO donors can also regulate a NO based pathway, i.e. improve drug safety and efficacy.

Classification of all NO donors could be confusing, since all nitrogen-oxygen-bonded compounds have the potential to decompose, be oxidized, or be reduced to produce reactive nitrogen species. However, similar chemical structures usually have a similar NO-releasing mechanism, so NO donors could be summarized in few groups according to their chemical classification:

3.5.1. Direct NO donors

Direct donors release NO spontaneously, through thermal or photochemical self-decomposition of a nitroso or nitrosyl functional group. This class of agents includes NO gas, sodium nitroprusside, sodium trioxodinitrate, S-nitrosothiols and diazeniumdiolates.

NO gas is used by inhalation for pulmonary vascular disorders. **Sodium nitroprusside (NP)** contains the nitric oxide group linked to iron and is used for the treatment of hypertension and heart failure in emergency [53]. To release NO, SNP requires either irradiation with light or one-electron reduction.

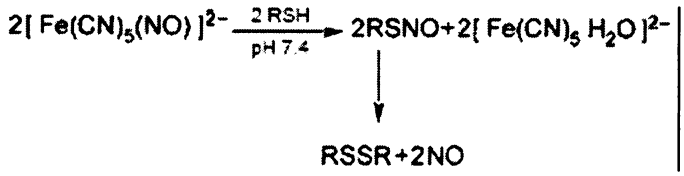

Fig. 5 *NP does not liberate NO spontaneously in vitro. It requires partial reduction (one-electron transfer) for example thiols.*

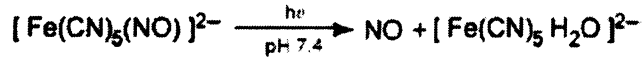

Fig. 6 *NP is also decomposed to NO by light (hv).*

A membrane-bound enzyme may be involved in generation of NO from the vasodilator in tissues. In a biological environment NP react with thiols to form an iron-nitrosyl-thiol complex. Further attack of thiolate anions may lead to decomposition of this complex with formation of disulfide, NO and cyanide. For clinical administration, cyanide toxicity may be a complication with long-term use. The class of NONOates or diazeniumdiolate compounds contains NO covalently linked to diethylamine and diethylenetriamine [54]. Sydnonimines and furoxan compounds require oxidants, such as molecular oxygen or thiols, to facilitate NO release [55]. S-nitrosothiols, including **S-nitrosoglutathione (SNOG)**, **S-nitroso-N-acetylpenicillamine (SNAP)** and S-nitrosoalbumin, release nitric oxide, induce transnitrosilation and S-thiolation [56]. S-nitrosylated low molecular thiols and protein thiols have been detected in biological fluids as consequence of endogenous metabolism and administration of NO Donors.

Since the transfer depends on the pKa of thiols, reaction proceeds at physiological pH levels. While most of the *S*-nitrosothiol compounds are unstable, SNAP and SNOG are exceptionally stable.

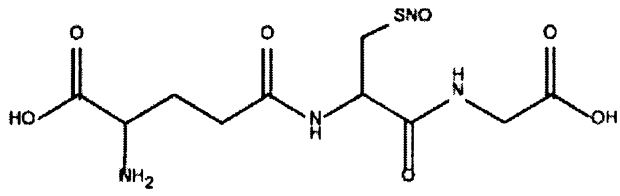

Fig.7 *Structure of SNOG.*

SNOG induces smooth muscle relaxation, vasodilatation and inhibits platelet activation *in vivo.* SNOG is an antioxidant that annihilates free radicals and promotes neuroprotection via its c-GMP independent nitrosylation action [57] and induces apoptosis in human adenocarcinoma cells in the presence of Cu and Ni ions [58] and also acts as an inhibitor of HIV-1 protease.

SNAP is a stable *N*-nitrosothiol commonly used as a NO donor. It was reported that the spontaneous liberation of NO from SNAP could not account for *in vitro* vascular relaxation. The spontaneous release of NO from nitrosothiol compounds may not be a key element of vascular relaxation; either metabolites of nitrosothiol generated at the cell membrane might be the essential element for relaxation [59].

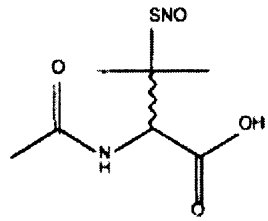

Fig. 8 *Structure of SNAP.*

3.5.2. Indirect NO donors

Indirect NO donors such as organic nitrates, nitrites and syndnonimines produce NO in chemical reaction with acid, alkali, metal and thiols, require metabolic activation, or both. These classical nitrovasodilators, used in the management of cardiovascular disorder, include

amyl nitrite, isosorbide dinitrite, isosorbide-5-mononitrate, nicorandil and organic nitrate and nitrite esters like GTN [60]. Nicorandil is an antianginal drug, which has the properties of both K^+ channel openers and NO donors. Structurally, it is a nicotinamide derivative with a nitrate group in its chemical structure. Alkyl- and aryloximes, which can tautomerize to C-nitroso compounds, are oxidatively converted to NO in vascular tissue [III]. As previously demonstrated [IV], inhibitors of soluble guanylyl cyclase and cGMP-dependent protein kinases abolished the endothelium independent relaxation of rat aortic rings mediated by oximes.

Potency of oximes in vasodelating studies with aortic rings is rather weak with exception of **formamidoxime** (FAM), **acetaldoxime** (AO) and especially **formaldoxime** (FAL). Nitric oxide production from FAL was inhibited by 7-ethoxyresorufin (ER) (an substrate of P450 1A1 and various NADPH-dependent reductases). It was subsequently reported [II] that one of the participating enzymes is diaphorase (DP). Besides FAL, DP was found to convert GTN. DP, aldehyde dehydrogenase (ALDH) [I] and glutathione-S-transferase [61, 62] and probably cytochrome P450 system involve the enzymatic denitration and reduction of organic nitrate esters. GTN, as the most important and well-characterized NO donor, will be described in a separate paragraph.

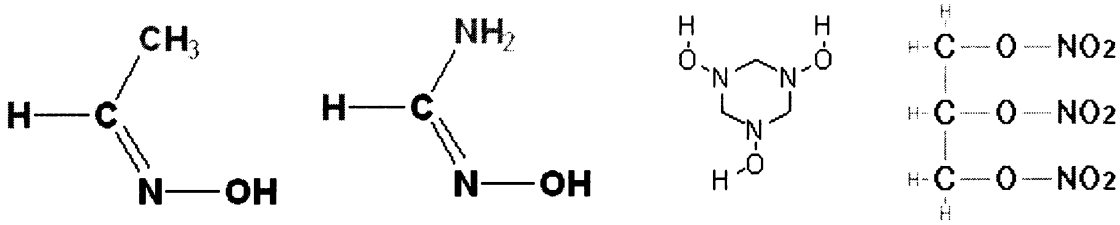

Fig. 9 *Structures of AO, FAM, FAL and GTN.*

3.5.3. Compounds that stimulate NO biosynthesis

Some compounds, like estrogen, statins and essential fatty acids have the ability to augment NO synthesis [63]. Angiotensin-converting enzyme inhibitors (ACEi) increase NO levels by decreasing the synthesis of angiotensin II and by enhancing of bradykinin levels. Antioxidants may also enhance NO biosynthesis [64].

3. 5.4. NO-drug hybrid molecules

Attaching a NO functional group to an existing drug has produced a novel class of antithrombotic agents. This innovative approach can offer various drug actions with synergistic effects, with reduced toxicity and side effects. For example, in 1999 a NO-releasing derivative of acetylsalicylic acid, NCX-4016, was developed, which is claimed to be able to overcome the major drawback associated with the use of aspirin as a pain reliever. NCX-4016 also shows a broader mechanism than aspirin and can inhibit additional inflammatory mediators (www.nicox.com) [65].

Based on the reports, that superoxide involves the mediation of tolerance, a bifunctional superoxide dismutase-mimic NO donor was designed in 2000 by Haj-Yehia's group [66]. It's the first compound that can simultaneously generate NO and destroy superoxide, which may lead to novel nontolerance-inducing nitrovasodilators [67].

3.5.5. GTN

The best known NO donor, GTN, has been used therapeutically for the treatment of angina pectoris, hypocardial infarction and congestive heart failure for over 130 years. [68, 69] NO production from GTN has been measured in cells, cellular fractions of vascular origin *in vitro*, blood, liver and other organs [70-73], and exhaled air following intravenous administration [74]. The mechanisms underlying GTN-induced smooth muscle relaxation and the development of nitrate tolerance during chronic treatment are still not completely understood. It is now clear, however, that the cellular effects of GTN include activation of the intracellular NO receptor enzyme, sGC thus increasing intracellular cyclic guanosine-3′-5′-monophosphate (GMP) levels [75, 76]. Interestingly, the smooth muscle relaxation by NO and GTN is partly mediated by a direct effect on ion channels [77-79]. Thus, GTN and other organic nitrates are believed to use the same signalling mechanism as endogenous NO generated by NOS.

All organic nitrates require either enzymatic or nonenzymatic bioactivation. Nonenzymaticaly is NO formed after interaction of nitrate esters with thiol groups . This adducts are oxidized to disulfides and nitrite (NO^{2-}) as the major metabolite [80]. Several other enzyme systems have been proposed to account for the bioactivation of organic nitrates [81-86] and candidate enzymes include glutahione S-transferases, cytochrom P450, cytochrome P450 reductase and xanthine oxidoreductase (XO), DP and ALDH.

3.6. Enzymes related to metabolism of NO-donating compounds

The mechanisms underlying enzyme metabolism of NO releasing agents are not completely understood. According to the hypothesis currently favored, NO donors undergo bioconversion in endothelial and smooth muscle cells into their putative metabolite, nitric oxide. Due to structural dissimilarities of NO donors, a variety of enzymes being responsible for bioactivation are currently suggested. Following enzymes or enzyme systems are supposed to be involved in biotransformation:

- **Cytochrom P450, CYP P450 reductase and related enzymes -** NADPH-cytochrom P 450 reductase is a simple flavoenzyme**,** one electron transferase, transforms organic nitrates, GTN [86, 61]
- **Glutathion S-transferase** – flavoenzyme; possesses diaphorase activity, biotransformation of GTN was descriebed [81, 87].
- **NAD(P)H oxidase** – Muenzel et al. (1995) reported biotransformation of oragnic nitrates [88].
- **XO** is a metal-containing flavoprotein, contains molybdenum and iron-sulfur centers in addition to the FAD prosthetic group. (see paragraph 3.6.4.)
- **Catalase/ Peroxidase** may transform hydroxylamine and *N*-Hydroxylnitrosamines [89].
- **NOS** is a heme-containing flavoprotein/ flavocytochrome that contains FAD, FNM and a heme prosthetic group with cytochrome P450-like characteristics, transforms guanidine and N-Hydroxyguanidine [89].
- **GC** (see paragraph 3.3.)
- **Mitochondrial ALDH** (see paragraph 3.6.7.)
- **Diaphorase** (see paragraph 3.6.1.)

At the first sight, some of these enzymes have common or related properties, like belonging to a flavoprotein superfamily or having a diaphorase activity.
"Diaphorase" is a bit confusing name, which has been loosely applied to several enzymes that catalyze the oxidation of either β-NADH or β-NADPH in the presence of an electron acceptor such as methylene blue or 2,6-dichlorophenol-indophenol.

Massey et al. [90] first described this reaction on a pig heart enzyme, isolated by Straub *et al.* in 1939 [91], later renamed to dihydrolipoamid dehydrogenase (LAD, see paragraph 3.6.2.). Massey than reported that denatured LAD is a DP [92].
The role of this enzyme in biotransformation of some NO donors was suggested by Chalupsky *et al.*[II].

Flavoproteins are biological electron acceptors and donors in flavin-mediated reactions can be two-electron acceptors or a variety of one-electron acceptors systems, such as NAD^+ cytochromes (Fe^{2+}/Fe^{3+}) and quinones. Molecular oxygen is an electron acceptor as well as the source of oxygen for oxygenase activity.
The reduced form ($FADH_2$) spontaneously reacts with O_2 to produce H_2O, but in dehydrogenase-like reaction, this reduced intermediate was not detected, or is somehow prevented from reacting with O_2.

There is a little evidence about the activity or substrate specificity of DP isolated from different species.
Currently, there are two commercially available products: Diaphorase from porcine heart and from *Clostridium kluyveri.*

3.6.1. DP from *Clostridium kluyveri*

Although diaphorases extracted from mammalian sources have been found to be components of the mitochondrial Complex I system, most of those of bacterial origin have yet to be assigned a physiological role.
DP from anaerobic bacterium *Clostridium kluyveri* is reported to be the only exception, which can not transfer electrons to oxygen and form peroxide even in the presence of flavin [93]. This was dismissed in a voltammetric, spectrophotometric and NMR experiments where only deaerated solutions before and during experiments showed no interference with molecular oxygen [94].
The previous results of our laboratory showed, that DP from *Clostridium kluyveri* is able to convert GTN and FAL to nitrites and small amount of nitrates with nitric oxide as an intermediate. Presence of NO was proven by electron paramagnetic spectroscopy. The reaction was inhibited by DPI (decrease of diaphorase activity of about 25% with GTN and 3% with FAL), and 7-ethoxyresorufin inhibited only the reaction with GTN. The reaction of

FAL produced decreased levels of nitrites (38%), when superoxide dismutase (SOD) was added, showing that reaction is probably influenced by simultaneous production of superoxide ion and relative species. Reaction of FAL was described as an oxidation in the presence of O_2 and simple reduction in the case of GTN [II].

3.6.2. LAD

Dihydrolipoamide dehydrogenase (Protein-N-(dihydrolipoyl)lysin:NAD^+ oxidoreductase, EC 1.8.1.4., formerly EC 1.6.4.3) is a part of a multienzymatic complexes: pyruvate dehydrogenase complex (PDC), 2-oxoglutarate complex and glycin decarboxylase complex. PDC of approximate molecular mass $8x10^6$ Da consists of multiple copies of three catalytic enzymes known as pyruvate dehydrogenase (E1), dihydrolipoamide acetyltransferase (E2) and dihydrolipoamide dehydrogenase (E3) [95]. Mutations to this homodimeric flavoprotein cause the often fatal human disease known as E3 deficiency.

LAD catalyses the reversible oxidation of NADH by lipoamide and contains a single molecule of non-covalently bound FAD and redoxactive disulfide in the active center. During the reaction NADH reduces FAD, which further (within milliseconds) transfers the electrons to the disulfide, forming a charge-transfer complex. The limiting step of catalysis is the oxidation of reduced disulfide by lipoamide [96].

$$NADH + H^+ + Lip(S)_2 \Leftrightarrow NAD^+ + Lip(SH)_2$$

Besides the active center disulfide, the native protein from pig heart contains eight cysteine residues per subunit as free thiols.

3.6.2.1. NADH oxidase activity

LAD, like many flavoproteins, can oxidize NADH by reducing molecular oxygen to H_2O. This intrinsic NADH oxidase activity is usually much slower than the dihydrolipoamide dehydrogenase activity, but is markedly increased (about 15-fold) in presence of KI, Zn or $(NH_4)_2SO_4$ and at the same time, the induced activity is less sensitive to pH, particularly on the alkaline side of the optimal pH. The effects of KI and $(NH_4)_2SO_4$ on catalytic activity is different. The NADH: lipoamide and NADH: *p*-benzoquinone reductase reactions of the enzyme were inhibited by KI, but both were accelerated by $(NH_4)_2SO_4$. KI may increase the oxidase activity by increasing both the reducibility of the flavin and the susceptibility of the reduced flavin to molecular oxygen [97].

3.6.2.2. Sensitivity to trace metals

Further studies of this enzyme have shown a sensitivity to trace metals. Preincubation with Cu^{2+} reduces the lipoamide dehydrogenase activity and proportionately increases the β-NADH diaphorase activity. It was demonstrated that this copper effect to some degree on the pig heart enzyme, but no appreciable effect was observed on the *Clostridium kluyveri* or torula yeast preparations. Massey *et al.* reported that the lipoamide dehydrogenase: diaphorase ratio could be a measure of denaturation of the enzyme [98]

3.6.2.3. pH

Dynamic light scattering studies of native pig heart LAD in aqueous solution indicated that dimer and tetramer state were preferred at both pH 5.8 and 7.5 and upon acidification to pH 5.8 only a monomer state with diaphorase activity was observed. This result may explain the reduced mitochondrial defenses and increasing oxidative stress that are observed during ischemia-reperfusion injury [99].

3.6.2.4. Structure

LAD belongs to the flavoprotein disulfide reductase protein family.

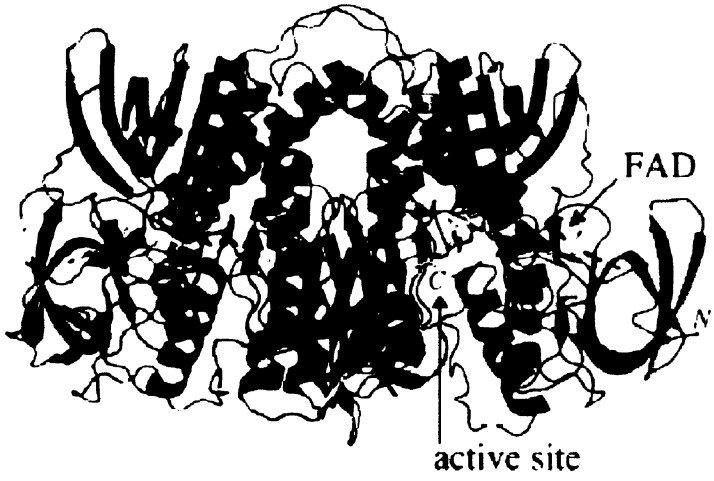

Fig. 10 *Structure of LAD from M. tuberculosis. Ribbon representation of LAD dimer. Protomers are colored blue or red. N and C demarcate the blue and red polypeptide termini, respectively. FAD and the active site are indicated by arrows and labeled.*

Members of the flavoprotein disulfide reductase family share low sequence identity (25–35%).

The amino acids sequence of LAD aligns well along the entire polypeptide chain only with glutathion transferase and trypanothione reductase [100, 101].

The structure of human LAD has also been recently reported [102]. On the basis of amino acid sequence and X-ray data is also a structural similarity to another disulfide-containing flavoprotein, glutathione reductase obvious.

Sequence alignment and sequence identity matrix for lipoamide dehydrogenase protein family members has shown that between bacterial and eukaryotic LAD is cca. 30%, between human, pig and dog about 90% homology [103].

3.6.2.5. NO scavenging

Igamberdiev *et al.* recently reported the capability of LAD to use NO as an electron acceptor, with NADH as the electron donor, forming nitrate in the reaction. Perhaps both FAD and disulfide are reduced during the reaction, forming a four-electron-reduced state of the enzyme. The importance of this reaction is that DPI did not inhibit the conversion. This result contributes to the suggestion that all DPI-inhibited reactions of flavoenzymes transfer one electron. While the K_m for NO is low, it is in the physiological range of NO levels encountered in the tissue. The NO conversion is pH-dependent, with highest K_m between pH 5-6; the enzyme may therefore have a significant role in modifying NO concentration under specific cell conditions like nitrosative stress. The NO scavenging activity was also over 90% inhibited by 1 $mmol.l^{-1}$ N-ethylmaleimide. KCN, DPI and antimycin A had no inhibitory effect [104].

3.6.2.6. Production of reactive oxide species (ROS)

Starkov *et al.* described that the flavin of LAD, like reduced flavins [105] and flavoproteins [106], can generate superoxide in aqueous solutions. This is of particular interest with regard to the mechanism and sites of ROS production in mitochondria, because the flavin of the LAD subunit has a sufficiently negative redox potencial to produce superoxide [107].

3.6.2.7. Inhibitors of LAD

Nonspecific inhibitors i.e.: arsenite, folic acid, iodoacetic acid, biopterine, carmustine (1,3-bis(2-chloroethyl)-1-nitrosurea). The rate of dihydrolipoamide oxidation is dependent upon NAD^+ / NADH ratio; the reaction is activated at high and inhibited at low ratio [108].
Pteridines were reported to inhibit dihydrolipoamide dehydrogenase activity of LAD from pig brain; transhydrogenase and diaphorase activities remained unaffected [109]. Currently, there is no evidence about a specific inhibitor.

3.6.3. DT-Diaphorase

The enzyme DT-diaphorase (NAD(P)H: (quinone acceptor) oxidoreductase, EC 1.6.99.2.) is a cytosolic flavoprotein, which catalyzes two or four electron reductions of a wide range of substrates (various quinones and oxidation-reduction dyes) using NADH or NADPH as electron donors. DT-Diaphorase functions via a ping pong bi-bi kinetic mechanism involving oxidized and reduced flavin forms of the free enzyme. Dicumarol (3, 3′-methylene-bis (4-hydroxycoumarine); DC), a potent inhibitor, binds to the oxidized form of the enzyme, competitively versus NADH [110]. Another inhibitors are 1-phenyl-3-pyrazolidinone (PH), capsaicin, and caffeine [111].

3.6.4. XO

The molybdoenzyme flavoprotein, xanthine oxidoreductase (XO, 126 kDa), is interesting in view of the ability of the dehydrogenase form (145 kDa) to catalyse the oxidation of NADH by oxygen, generating superoxide anion and hydrogen peroxide. NAD^+ and diphenyliodonium inhibited NADH oxidation and superoxide production. Established inhibitors of XO (allopurinol, oxypurinol and amflutizole), which block all other reducing substrates were ineffective in the case of NADH [112].
XO may also be a contributory source of NO especially in hypoxic conditions, when NOS cannot generate NO [83]. Doel *et al.* suggested, that XO has both inorganic nitrate reductase and nitrite reductase activities at its Mo-Co site. Further, they suggested that NO may be generated by XO via the reduction of organic nitrates like isosorbide dinitrate and GTN and organic nitrites e.g. isoamyl nitrite.

Doel *et al.* (2001) demonstrated that reduction of organic nitrates occurs at the FAD site [113], in contrast to the reduction of inorganic nitrate at Mo-Co [114]. Li *et al.* concluded from studies using NO chemiluminiscence and NO analyzer, that organic nitrite (R-O-NO) is produced from XO-mediated organic nitrate reduction. Organic nitrite further reacts with thiols and leads thus to NO release and to stimulation of sGC [115].
This conclusion could be theoretically applied on reactions of DP with organic nitrates, because results of Bartik *et al.* have confirmed the presence of NO with EPR spectroscopy [II.] but still, it does not exclude a preceding reaction of nitrites with thiols.

3.6.6. Reaction of reduced flavins with GTN

GTN denitration could be carried out only by reduced flavins. Thus, when $FADH_2$ was allowed to react anaerobically with GTN, more than 90% of GTN was reoxidized with approximately one-third being oxidized with $t_{1/2}$ of 30 s, another third with $t_{1/2}$ of $\approx$ 10 min and the remainder with $t_{1/2} \approx$ 140 min. These results of Meah *et al.* show that all three nitrate ester groups can be reduced by the flavin, with the first reacting $\approx$ 20 times faster than the second, which in turn is considerably more reactive than the third [116].

3.6.7. Mitochondrial ALDH

Mitochondrial ALDH (EC 1.2.1.3) was identified as an enzyme playing a central role in GTN biotransformation, generating 1,2-glyceroldinitrate, 1,3-glyceroldinitrate, inorganic nitrate and NO in tissues [117]. Recent experimental work has defined new tolerance mechanisms, including the inhibition of mitochondrial ALDH isoform 2 by GTN-induced ROS [118].

3.7. Inhibitors used in experiments:

3.7.1. DPI

Dibenziodolium chloride
DPI is a potent flavoprotein inhibitor. Several vascular and nonvascular flavin-containing enzymes that are inhibited by DPI have been identified, e.g. NADPH-cytochrome P 450 reductase [61], neutrophil NAD(P)H oxidase [119], xanthine oxidase/dehydrogenase[120],

mitochondrial NADH-ubiquinone oxidoreductase[121] and NOS [122]. It irreversibly inhibits endothelium-dependent vasodilations in a similar manner to the NG-substituted arginine analogs, but this effect is attenuated or abolished by blockers of the sympathetic nervous system and does not lead to a sustained rise in blood pressure.

DPI targets enzymes that are involved in the biotransformation of organic nitrates and that are involved in the vasodilator response to organic nitrates. It was reported that DPI caused a decreased 1,2-glyceroldinitrate formation in isolated rat aorta. DPI also inhibits the hemodynamic response to GTN in an in vivo rat model [123].

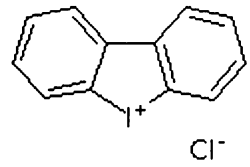

Fig. 11 *Chemical structure of DPI.*

3.7.2. Dicoumarol (DC)

3,3′-methylene-bis (4-hydroxycoumarin)

DC is a coumarin-like compound found in sweet clover. It is used in biochemical experiments as an inhibitor of reductases, as an oral anticoagulant. It acts by inhibiting the hepatic synthesis of vitamin K-dependent coagulation factors [124].

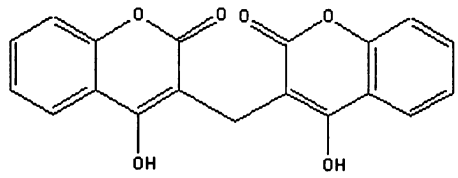

Fig.12 *Chemical structure of DC.*

3.7.3. ER

Resorufin ethyl, 7-Ethoxy-3H-phenoxazin-3-one

Specific substrate for cytochrome P450 isozyme: CYP1A1 (CXPIA1/2)

Resorufin-based cytochrome P450 substrates are substrates for microsomal dealkylases acetyltransferases, luciferases and other enzymes. Resorufin ether substrates, which all yield

fluorescent resorufin, have been extensively used to differentiate isozymes of cytochrome P450 [125].

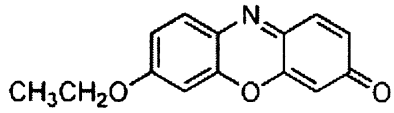

Fig. 13 *Chemical structure of ER.*

3.7.4. Phenidone (PH)

1-Phenyl-3-pyrazolidinone

Phenidone is dual lipoxygenase and cyclooxygenase inhibitor [126] and inhibitor of D,T-diaphorase [111].

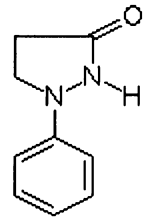

Fig.14 *Chemical structure of PH.*

3.7.5. L-NAME

An analog of arginine that inhibits NO production by inhibiting nitric oxide synthase. It has multiple effects on the vascular system. It inhibits relaxation induced by acetylcholine and induces an increase in arterial blood pressure [127]. It abolishes lecithinized superoxide dismutase induced vasodilation when used to pretreat aortic ring preparations of mice [128]. It induces leukocyte adhesion [129], increases microvascular fluid [130], protein fluxes and permeability [131].

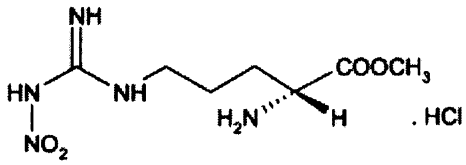

Fig. 15 *Chemical structure of L-NAME.*

3.7.6. Proadifen hydrochloride (PA)

α-Phenyl-α-propylbenzeneacetic acid 2-(diethylamino)ethyl ester; N,N-diethylaminoethyl 2,2 diphenylvalerate ; 2-[diethylamino]ethyl ester hydrochloride

Proadifen is a cell permeable reversible inhibitor of cyrochrome P450. It inhibits neuronal nitric oxide synthase and stimulates endothelial cell prostacyclin production while inhibiting platelet thromboxane synthesis [132].

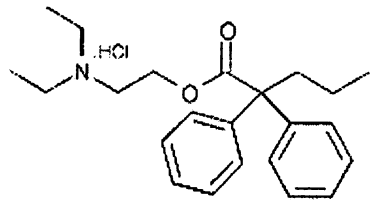

Fig. 16 *Chemical structure of PA*

3.7.7. Miconazole (MC)

1-(2,4-Dichloro-β-[(2,4 dichlorobenzyl)oxy]phenethyl)imidazole
Miconazole is an azole inhibitor of cytochrom P450 3A4.

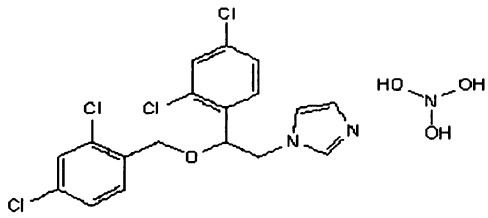

Fig. 17 *Chemical structure of MC.*

4. MATERIALS

Chemicals used:

Diaphorase from *Clostridium kluyveri*, N^{ω}-nitro-L-arginine methyl ester (L-NAME), 7-ethoxyresorufin, dihydrolipoamide dehydrogenase from porcine heart, dimethylsulfoxide, diphenyleneiodonium chloride, sulfanilamide, NADH, Trizma Base, crystalline bovine serum albumin, formamidoxime, 3,3′-methylene-bis(4-hydroxycoumarine), miconazole nitrate salt, 4-methylpyrazole chloride, 1-pheny-3-pyrazolidinone were obtained from Sigma (Aldrich). Sodium nitroprusside was purchased from Lachema and N-(1-naphthyl)-ethylenediamonnium dichloride from Riedel-de Haeen. Acetaldoxime, glycerol trinitrate were purchased from Merck. Formaldoxime hydrochloride (triformoxime hydrochloride) from Fluka.

5. METHODS:

As described, enzymes used in tests are diaphorases (1.4.1.8.). In order to distinguish the origin and different properties of the enzyme, enzyme from *Clostridium kluyverii* is indicated in text as DP and enzyme from Porcine heart as LAD.

Reactions were performed under non-saturating conditions.
All concentrations in text are expressed as final concentrations in reaction mixture.

5.1. Determination of nitrites

Nitric oxide release was indirectly determined by using the diazo-coupling method. Main of its stable decomposition endproduct nitrite directly reacts with the Griess reagent: 1:1 solution of 1% (w/v) sulfanilamide and 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride in 2.5% (v/v) orthophosphoric acid [132] to form a purple chromophoric azo compound. An aliquot of the sample was mixed with fresh reagent at the room temperature. Absorbance was measured at 540 nm 10 min. later with a spectrophotometer to get the nitrite concentration. The detection limit for the assay is about 1μmol/l.

5.2. Preparation of the nitrite standard curve

The stock solution was prepared by dissolving sodium nitrate in Tris-HCl buffer pH 7.5 (0.05 $mmol.l^{-1}$). Serial dilutions were then made to provide the five final solutions in triplicate. The buffer alone was used as a control in each assay.

5.2.1. Effect of $(NH_4)_2SO_4$ on the standard curve

LAD is dissolved in 2.9 mmol/l $(NH_4)_2SO_4$. To exclude the interference of ammonia ions with Griess assay, corresponding serial dilutions of ammonia ions as in samples were prepared (2.5-20 μmol/l). The Griess assay was applied.

5.3. Enzymatic transformation of NO donors

Some of vasodilating compounds used in tests are light sensitive, or decompose spontaneously in solutions. Fresh solutions were therefore prepared before every experiment and were kept in dark. Degradation was corrected by measurement of control samples. Because of sensitivity of enzymes to metal ions, only redistilled water was used in all experiments. The water was also free of nitric ions.

5.3.1. Preliminary tests

Time-dependence of reactions

In order to find the initial rate of reactions, where nitrite production is continuous and linear and to find an optimal relation between amount of enzyme vs. substrate, preliminary tests were run. Samples were incubated in dark at 37°C.

Reaction mixtures contained 0.728 U/ml DP, 1mmol/l NADH, and one of following substrates (2 mmol/l GTN, 10 mmol/l FAM, 10 mmol/l AO, 10 mmol/l FAL, 0.25 mmol/l NP, 0.5 mmol/l SNOG or 0.1 mmol/l SNAP) in 0.05 mol/l Tris-HCl buffer (pH 7.5). The control samples were without enzyme. Aliquots for Griess assay (paragraph 5.1.) were taken after 5, 15, 45, 60, 120 and 180 minutes.

The LAD assay contained 0.728 U/ml LAD, 1mmol/l NADH and one of following substrates (2 mmol/l GTN, 10 mmol/l FAM, 10 mmol/l AO, 10 mmol/l FAL, 0.025 mmol/l NP, 0.5 mmol/l SNOG or 0.5 mmol/l SNAP). Aliquots for Griess assay (paragraph 5.1.1) were taken after 30, 60, 90, 120 and 180 minutes. All samples were measured in triplicates.

5.3.2. Kinetic parameters of reactions with DP

The reaction mixture (1 ml) in 0.05 mmol/l Tris-HCl buffer pH 7.5 containing DP from *Clostridium Kluyveri* (0.3 mg/ml, 5.1 U/mg of solid) and NADH (1 mmol/l) and selected NO donors (concentrations in Tab.1) were incubated at 37°C in dark for 60 min. An 0.1-ml aliquot of the incubated sample was removed in 10-min intervals and 0.1 ml of Griess reagent was added. The absorbance of the chromophore formed was measured at 540 nm Tests were performed in triplicate. The control samples were without enzyme.

Tab. 1 *Concentrations of substrates.*

Substrate:	GTN	FAL	AO	SNAP	SNOG	NP
Concentration ($\mu mol.l^{-1}$):	0.2-3.2	0.2.-3.2	0.6-9.6	0.05-0.8	0.05-0.8	0.1-1.6

5.3.3. Kinetic parameters of reactions with LAD

The reaction mixture (1 ml) in 0.05 mmol/l Tris-HCl buffer pH 7.5 containing LAD from porcine heart 2.9 U/ml (13.0 mg prot./ ml (biuret), 59 U/mg prot.), NADH (1 mmol/l) and selected NO donors (concentrations in Tab. 2) were incubated at 37°C in dark for 60 min. A

0.1-ml aliquot of the incubated sample was removed in 10-min intervals and 0.1 ml Griess reagent was added. The absorbance of the formed chromophore was measured at 540 nm Tests were performed in triplicate. The control samples were without enzyme.

Tab. 2 *Concentrations of substrates*

Substrate:	GTN	FAL	AO	SNAP	SNOG	NP
Concentration ($mmol.l^{-1}$):	0.2-3.2	0.2.-3.2	0.6-9.6	0.05-0.8	0.05-0.8	0.1-1.6

Constants V_{max} and K_m were estimated in experiments, in which concentration of substrate was varied and the rate, or the concentration of product resp., were measured after the time interval. The concentration range of substrates is given in the Tab. 2.

5.4. Inhibition assay

Since DPI, DC, MC and ER were dissolved in DMSO to a final concentration of less than 1%, samples in the control group were incubated in the presence of DMSO at the same concentration.

In order to determine whether the inhibitory effect could also be attributed to DMSO, samples with equal concentration of DMSO without inhibitor were compared.

5.4.1. Absorption spectrum of inhibitors

Samples with inhibitors of the highest concentration used in inhibition assays with or without NADH (0.875 mmol/l) were incubated in 100 µl 0.05 mmol/l Tris-HCl buffer pH 7.5. After 10 min, an aliquot of Griess reagent was added and the absorbance was measured as described.

5.4.2. Determination of K_i

DP (0.3 mg/ml, 5.1 U/mg of solid) or LAD (2.9 U/ml of solution) resp. were incubated in the presence of inhibitors (concentrations in Tab. 3) with one concentration of NO donors (concentrations in Tab. 4) in the at 37°C in dark for 60 min in 0.05 mmol/l Tris-HCl buffer pH 7.5. An 0.1-ml aliquot of the incubated sample was removed in 10-min intervals and 0.1 ml Griess reagent was added. The absorbance of the chromophore formed was measured at 540 nm. Tests were performed in triplicate. The control samples were without enzyme.

A Dixon graph of the reciprocal velocity against inhibitor concentration was then plotted.

The K_i of all inhibitors were calculated from the Dixon plot. For a classical noncompetitive inhibitor, the $-K_i$ gives the point where the lines intersect each other on inhibitor axis. For competitive, or mixed, inhibitors the intersection is above the axis.

Tab.3 *Concentrations of inhibitors.*

Inhibitor:	DPI	ER	PH	L-NAME	PA	MC
Concentration ($\mu mol.l^{-1}$):	10-500	7.5-75	25-250	25-250	25-250	25-250

Tab.4 *Concentrations of substrates*

Substrate:	GTN	FAL	AO	SNAP	SNOG	NP
Concentration ($mmol.l^{-1}$):	2	10	10	0.5	0.5	1

5.4.3. Determination of inhibition constants

The experiments were performed as described in paragraph 5.4.2., but fixed concentrations of inhibitors were added (Tab. 5) to different amounts of NO donors (Tab. 6).

Tab.5 *Concentrations of substrates*

Inhibitor:	DPI	ER	PH	L-NAME	PA	MC
Concentration ($\mu mol.l^{-1}$):	100	100	-	-	-	-

Tab.6 *Concentrations of substrates*

Substrate:	GTN	FAL	AO	SNAP	SNOG	NP
Concentration ($\mu mol.l^{-1}$):	0.2-3.2	0.2-3.2	0.6-9.6	0.05-0.8	0.05-0.8	0.1-1.6

After nonlinear regression, initial rates were calculated for each reaction. To determine enzymatic and inhibition constants, data were calculated with the use of nonlinear plot of Hyper 32 program (www.liv.ac.uk/~jse/software.html)

A plot of the reaction rate versus concentration of substrate was constructed for each inhibitor and Km was then calculated. The IC_{50} value were calculated from K_i and K_m.

$$K_i = \frac{IC_{50}}{1 + \frac{[Substrate]}{K_M}}$$

5.4.4. Oxidase activity

The oxidation of NADH (0.25 mmol/l) in the presence of LAD (2.9U/ml) or DP (1.7 U/ml) was followed at 340 nm for 5-10 min at laboratory temperature. Final volume of reaction mixture was 200 µl.

5.4.5. Nitrosylation reaction of SNAP and SNOG

To elucidate an nonenzymatic decomposition of substrates, which are able to react with thiol groups in proteins, assay with well known protein, bovine serum albumine (BSA) was done. The reaction mixture contained 0.1 mmol/l SNAP or 0.5 mmol/l SNOG, resp. 1mmol/l NADH and 0.1 mmol/l BSA. The control samples contained no BSA. Nitrites were measured as described in paragraph (5.1.).

6. RESULTS

All fitted regression curves shown on the figures are calculated by "least squares". For most of the data presented in this paragraph, the errors in the transfer velocity are expected to be similar for all measurements and can therefore be deduced from the data scattering. Therefore error bars are not included in most of the figures.

6.1. Determination of nitrites

Results are expressed as mean ± SEM of three experiments.

6.2. Nitrite Standard Curve

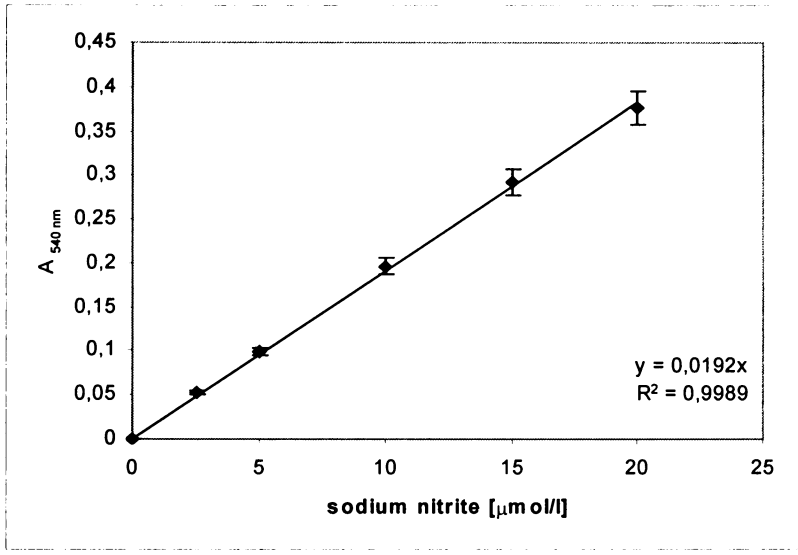

Fig. 18 *Standard curve for nitrite determination with Griess assay.*

6.2.1. Effect of $(NH_4)_2SO_4$ on the standard curve

The effect of ammonia ions in the concentration range similar to LAD samples was negligible. The difference between nitrite standard curve with and without added $(NH_4)_2SO_4$ was less than 2%.

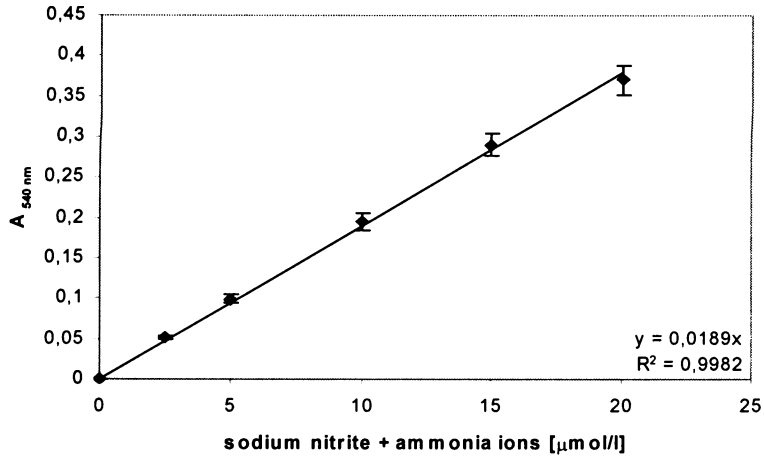

Fig. 19 *Effect of presence of $(NH_4)_2SO_4$ on the standard curve.*

6.3. Enzymatic transformation of NO donors

According to these preliminary tests, suitable substrate concentrations for further experiments were selected. One of the substrate, FAM was eliminated, because under experimental conditions, no enzymatic transformation was observed. Data not shown.

6.3.1. Kinetic parameters of reactions with DP

Kinetic parameters for reactions of DP with GTN, SNAP, SNOG, AO and FAL were obtained from initial velocities (Tab. 7-11). Time-course of NP reaction has prevented the determination of parameters (Fig. 30). As GTN produced the highest levels of nitrites, and FAL the lowest, the order of substrates is about GTN>SNOG>NP>SNAP>AO>FAL. On the other hand, the order according to kinetic parameters of hyperbolic regression is SNAP>GTN>AO>SNOG>FAL. V_{max} and K_m were calculated from four different types of plot, the linear Lineweaver-Burk plot shows the data.

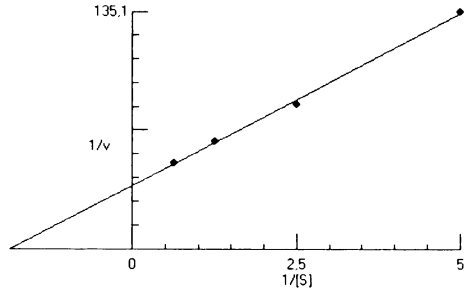

Fig. 20 *Lineweaver-Burk plot for reaction of DP with GTN. 1/v means reciprocal initial rate 1/[mmol.min^{-1}], 1/s means reciprocal concentration of substrate 1/[mmol. l^{-1}] for this reaction.*

Tab. 7 DP + GTN		
	Km (mmol)	Vmax (mmol.l^{-1} min^{-1})
Hanes plot results	0,51	0,02
Lineweaver-Burk plot results	0,54	0,03
Eadie-Hofstee plot results	0,51	0,03
Hyperbolic regression results weighted	0,52 ± 0,01	0,03 ± 0,00

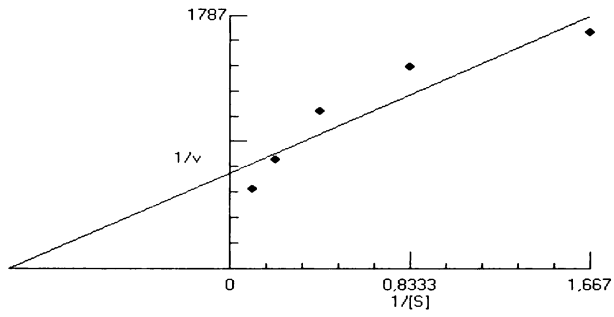

Fig. 21 *Lineweaver-Burk plot for reaction of DP with FAL. 1/v means reciprocal initial rate 1/[mmol.min^{-1}], 1/s means reciprocal concentration of substrate 1/[mmol. l^{-1}] for this reaction.*

Tab. 8 DP + FAL		
	Km (mmol)	Vmax (μmol.l^{-1} min^{-1})
Hanes plot results	2,61	2,197
Lineweaver-Burk plot results	0 ,99	1,481
Eadie-Hofstee plot results	1,25	1,664
Hyperbolic regression results weighted	1,53 ± 0, 22	1,72 ± 0,00

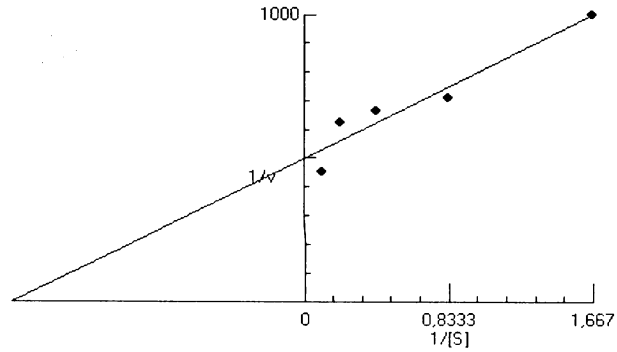

Fig. 22 *Lineweaver-Burk plot for reaction of DP with AO. 1/v means reciprocal initial rate 1/[mmol.min^{-1}], 1/s means reciprocal concentration of substrate 1/[mmol. l^{-1}] for this reaction.*

Tab. 9 DP + AO		
	Km (mmol)	Vmax (mmol.l^{-1} min^{-1})
Hanes plot results	1,19	0,002354
Lineweaver-Burk plot results	0,60	0,002003
Eadie-Hofstee plot results	0,63	0,002045
Hyperbolic regression results weighted	0,60 ± 0,06	0,00198 ± 6,01e-04

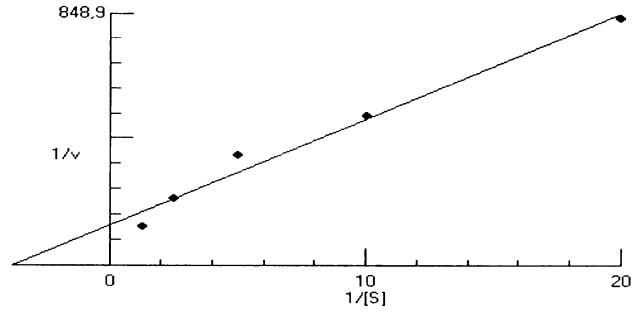

Fig. 23 *Lineweaver-Burk plot for reaction of DP with SNAP. 1/v means reciprocal initial rate 1/[mmol.min^{-1}], 1/s means reciprocal concentration of substrate 1/[mmol. l^{-1}] for this reaction.*

Tab. 10 DP + SNAP		
	Km (mmol)	Vmax (mmol.l^{-1} min^{-1})
Hanes plot results	0 ,61	0,01293
Lineweaver-Burk plot results	0 ,27	0,007481
Eadie-Hofstee plot results	0 ,37	0,009371
Hyperbolic regression results weighted	0,45 ± 0,06	0,01014 ± 0,00934

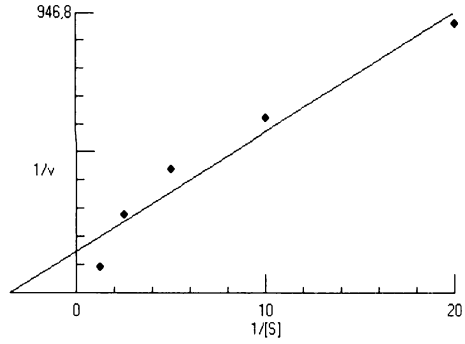

Fig. 24 *Lineweaver-Burk plot for reaction of DP with SNOG. 1/v means reciprocal initial rate 1/[mmol.min^{-1}], 1/s means reciprocal concentration of substrate 1/[mmol. l^{-1}] for this reaction.*

Tab. 11 DP + SNOG

	Km (mmol)	Vmax (mmol.l^{-1} min^{-1})
Hanes plot results	2,6	0,04098
Lineweaver-Burk plot results	0,29	0,007176
Eadie-Hofstee plot results	0,22	0,007458
Hyperbolic regression results weighted	0,82 ± 0,32	0,01437 ± 0,04389

6.3.2. Reactions with LAD

Enzyme kinetic data of GTN, SNAP, SNOG, AO and FAL decomposition by LAD are in Tab.12-17. LAD converted all selected substrates, but in comparison to DP, with a lower efficiency. According to NO release, the best substrates were decreasingly SNOG>SNAP>GTN>FAL>AO and according to K_m SNAP>AO>SNOG>GTN>FAL. Similar to DP, parameters for reaction with NP were not determined, the time-course of NP reaction has prevented the determination of parameters (Fig. 31).

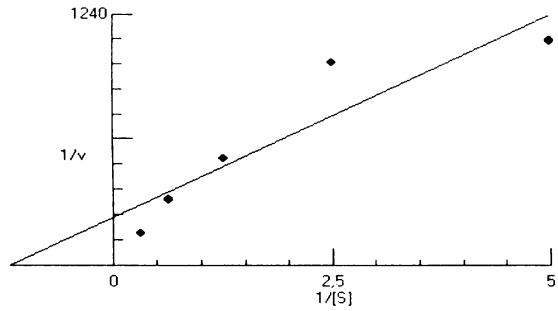

Fig. 25 *Lineweaver-Burk plot for reaction of LAD with GTN. 1/v means reciprocal initial rate 1/[mmol.min^{-1}], 1/s means reciprocal concentration of substrate 1/[mmol. l^{-1}] for this reaction.*

Tab. 12 LAD + GTN		
	Km (mmol)	Vmax (mmol.l^{-1} min^{-1})
Hanes plot results	4,50	0,01387
Lineweaver-Burk plot results	0,86	0,004271
Eadie-Hofstee plot results	1,25	0,005946
Hyperbolic regression results weighted	3,87 ± 1,04	0,01191 ± 0,02571

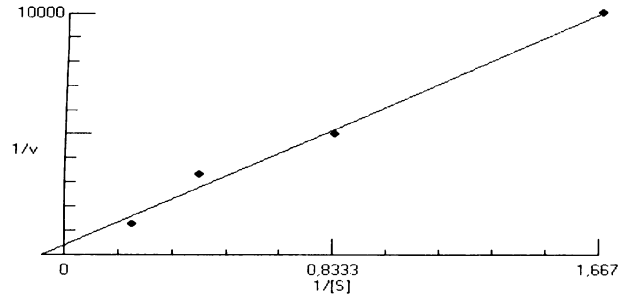

Fig. 26 *Lineweaver-Burk plot for reaction of LAD with FAL. 1/v means reciprocal initial rate 1/[mmol.min^{-1}], 1/s means reciprocal concentration of substrate 1/[mmol. l^{-1}] for this reaction.*

Tab. 13 LAD + FAL		
	Km (mmol)	Vmax (mmol.l^{-1} min^{-1})
Hanes plot results	222	0,0345
Lineweaver-Burk plot results	14,44	0,0025
Eadie-Hofstee plot results	-1,600e00	1,000e-04
Hyperbolic regression results weighted	128,6 ± 4,77e03	0,0198 ± 0,721

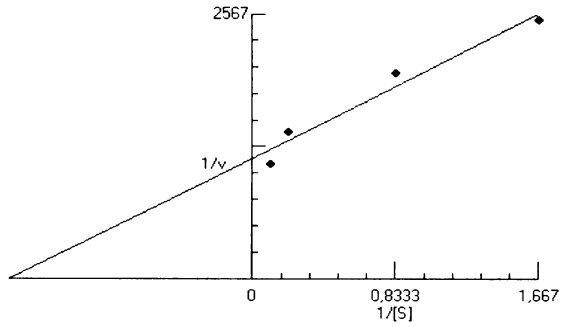

Fig. 27 *Lineweaver-Burk plot for reaction of LAD with AO. 1/v means reciprocal initial rate 1/[mmol.min^{-1}], 1/s means reciprocal concentration of substrate 1/[mmol. l^{-1}] for this reaction.*

Tab. 14 LAD + AO		
	Km (mmol)	Vmax (mmol.l^{-1}.min^{-1})
Hanes plot results	1,19	0,002354
Lineweaver-Burk plot results	0,60	0,002003
Eadie-Hofstee plot results	0,63	0,002045
Hyperbolic regression results weighted	0,60 ± 0,06	0,00198 ± 6,01e-04

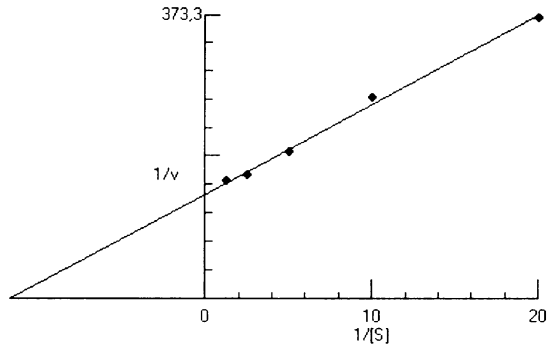

Fig. 28 *Lineweaver-Burk plot for reaction of LAD with SNAP. 1/v means reciprocal initial rate 1/[mmol.min^{-1}], 1/s means reciprocal concentration of substrate 1/[mmol. l^{-1}] for this reaction.*

Tab. 15 LAD + SNAP		
	Km (mmol)	Vmax (mmol.l^{-1} .min^{-1})
Hanes plot results	0,08	0,01293
Lineweaver-Burk plot results	0,27	0,007481
Eadie-Hofstee plot results	0,37	0,009371
Hyperbolic regression results weighted	0,45 ± 0,06	0,01014 ± 0,00934

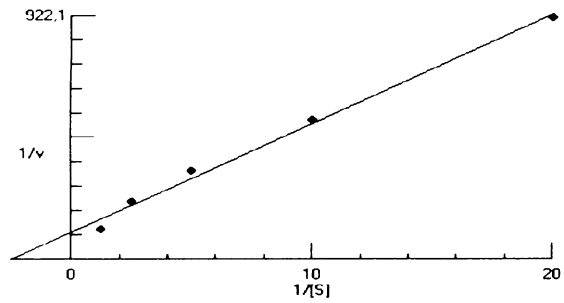

Fig. 29 *Lineweaver-Burk plot for reaction of LAD with SNOG. 1/v means reciprocal initial rate 1/[mmol.min^{-1}], 1/s means reciprocal concentration of substrate 1/[mmol. l^{-1}] for this reaction.*

Tab. 16 LAD + SNOG

	Km (mmol)	Vmax (mmol.l^{-1} .min^{-1})
Hanes plot results	2,64	0,04098
Lineweaver-Burk plot results	0,01	0,2897
Eadie-Hofstee plot results	0,22	0,007458
Hyperbolic regression results weighted	0,82 ± 0,32	0,01437 ± 0,004389

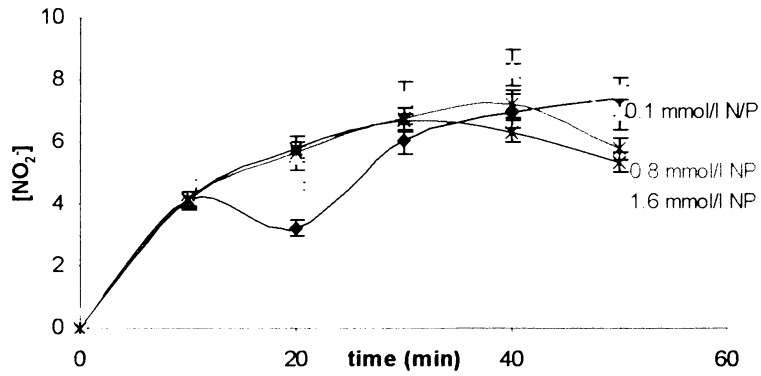

Fig. 30 *Nitrite production in reaction of DP with NP. Results are expressed as mean ± SEM (n=3).*

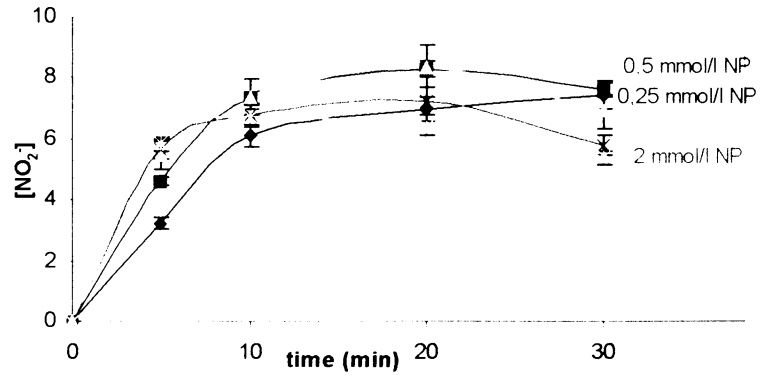

Fig. 31 *Nitrite production in reaction of LAD with NP. Results are expressed as mean ± SEM (n=3).*

Tab. 17 *Enzyme kinetic parameters of all reactions, obtained from hyperbolic regression.*

	DP		LAD	
	K_m (mmol)	V_{max} ($\mu mol.l^{-1}.min^{-1}$)	K_m (mmol)	V_{max} ($\mu mol.l^{-1}.min^{-1}$)
GTN	0,52 ± 0,14	27,01 ± 0,38	3,87 ± 1,05	11,91 ± 2,57
FAL	1,53 ± 0,22	1,7 ± 0,11	128,6 ± 4,77e03	19,79 ± 0,72
AO	0,60 ± 0,06	1,99 ± 6,01e-07	0,79 ± 0,09	8,7e-07 ± 3,6e-07
SNAP	0,44 ± 0,06	10,1 ± 0,93	0,09 ±0,02	7,36 ± 6,04e-04
SNOG	0,82 ± 0,32	14.37 ± 4,39	0,64 ± 0,08	13,7 ± 0,013

6.4. Inhibition assay

The presence of DMSO in samples in amount necessary for dilution of some inhibitors had no effect on enzymatic reactions. Data not shown.
The Overview of inhibition effects shows Tab. 25.

6.4.1. Absorption spectrum of inhibitors

Only DC and ER from all inhibitors used in assays have significantly increased the absorbance at 540 nm. The absorbance values of DC in a wide spectrum of concentrations (2.5-250 μmol/l) have disabled further experiments. Data not shown.

6.4.2. Determination of K_i

DPI inhibited transformation of all substrates in a concentration-dependent manner.
All reactions of GTN, AO, FAL, NP, SNAP and SNOG with DP or LAD resp. were inhibited by DPI . DPI inhibited the reactions with 42-55% efficiency according to control reaction without inhibitor.

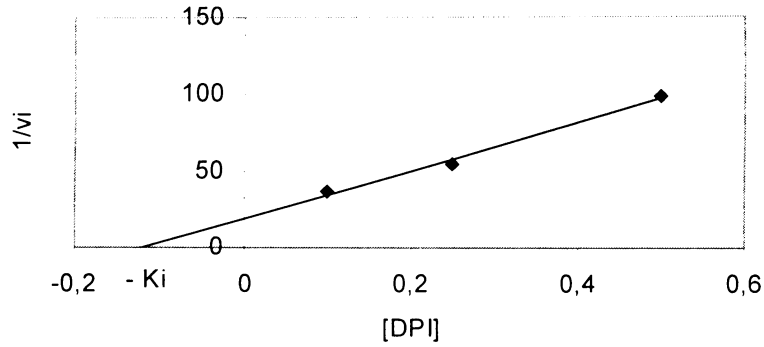

Fig. 32 *Dixon plot for reaction of DP with GTN in the presence of DPI. 1/v means reciprocal initial rate 1/[mmol.min^{-1}], [DPI] means concentration of inhibitor [mmol. l^{-1}] for this reaction. K_i is 0,12 mmol.l^{-1}.*

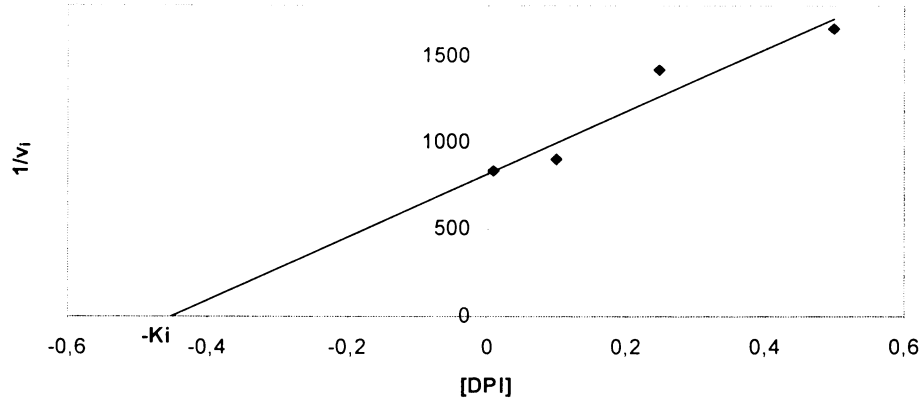

Fig. 33 *Dixon plot for reaction of DP with AO in the presence of DPI. 1/v means reciprocal initial rate 1/[mmol.min^{-1}], [DPI] means concentration of inhibitor [mmol. l^{-1}] for this reaction. K_i is 0,45 mmol.l^{-1}.*

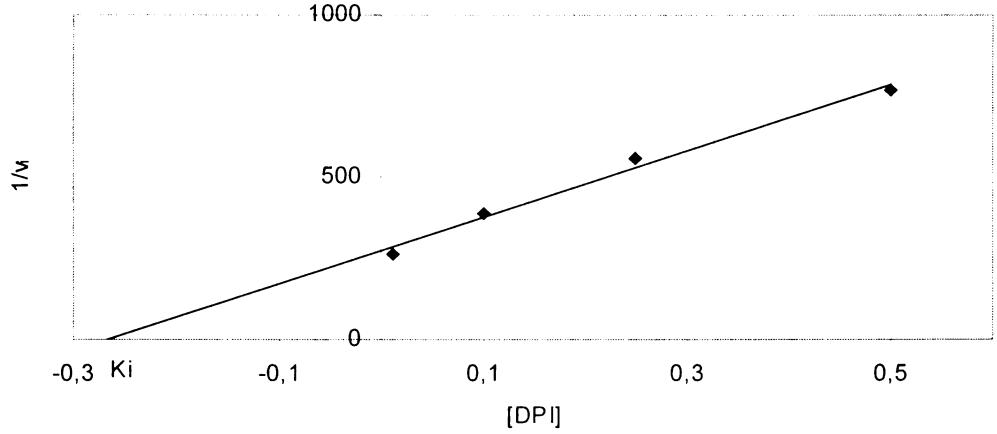

Fig. 34 *Dixon plot for reaction of DP with FAL in the presence of DPI. 1/v means reciprocal initial rate 1/[mmol.min^{-1}], [DPI] means concentration of inhibitor [mmol. l^{-1}] for this reaction. K_i is 0,27 mmol.l^{-1}.*

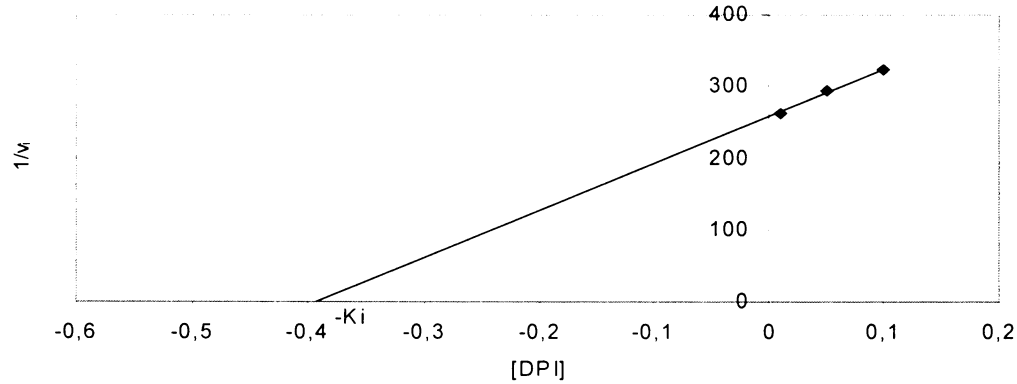

Fig. 35 *Dixon plot for reaction of DP with SNAP in the presence of DPI. 1/v means reciprocal initial rate 1/[mmol.min^{-1}], [DPI] means concentration of inhibitor [mmol. l^{-1}] for this reaction. K_i is 0,39 mmol.l^{-1}.*

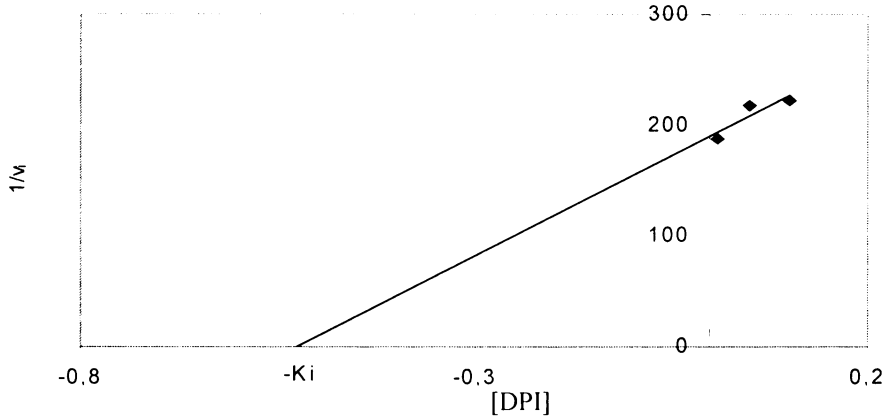

Fig. 36 *Dixon plot for reaction of DP with SNOG in the presence of DPI. 1/v means reciprocal initial rate 1/[mmol.min^{-1}], [DPI] means concentration of inhibitor [mmol. l^{-1}] for this reaction. Ki is 0,53 mmol.l^{-1}.*

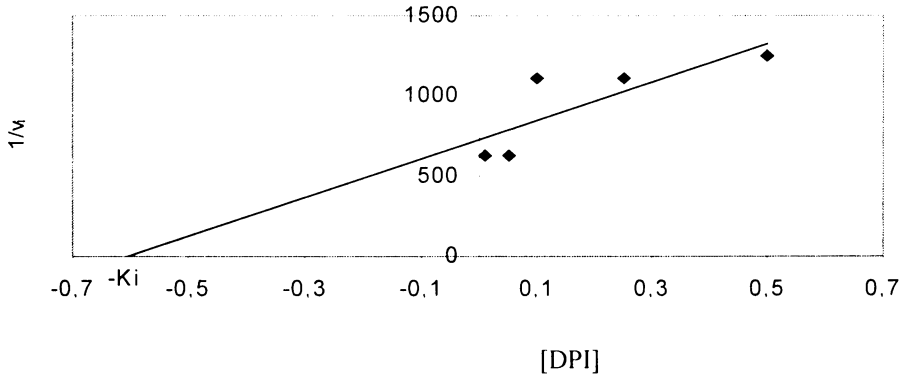

Fig. 37 *Dixon plot for reaction of LAD with GTN in the presence of DPI. 1/v means reciprocal initial rate 1/[mmol.min^{-1}], [DPI] means concentration of inhibitor [mmol. l^{-1}] for this reaction. Ki is 0,60 mmol.l^{-1}.*

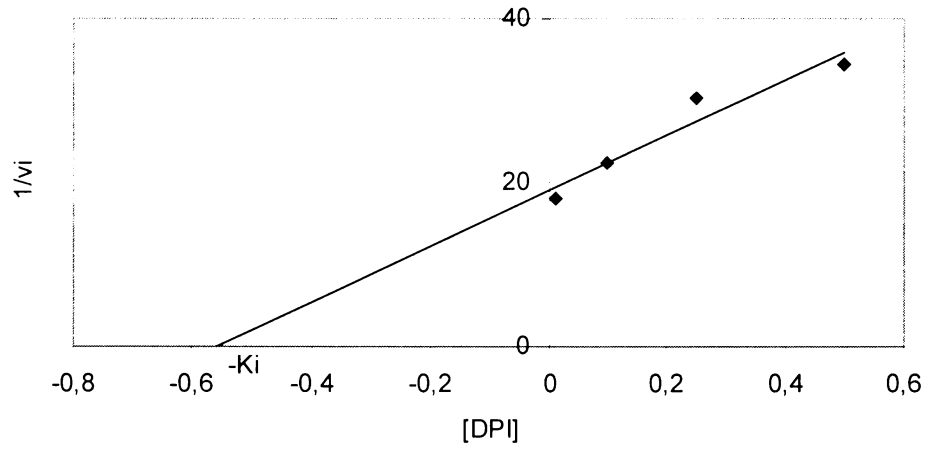

Fig. 38 *Dixon plot for reaction of LAD with FAL in the presence of DPI. 1/v means reciprocal initial rate 1/[mmol.min^{-1}], [DPI] means concentration of inhibitor [mmol. l^{-1}] for this reaction. Ki is 0,56 mmol.l^{-1}.*

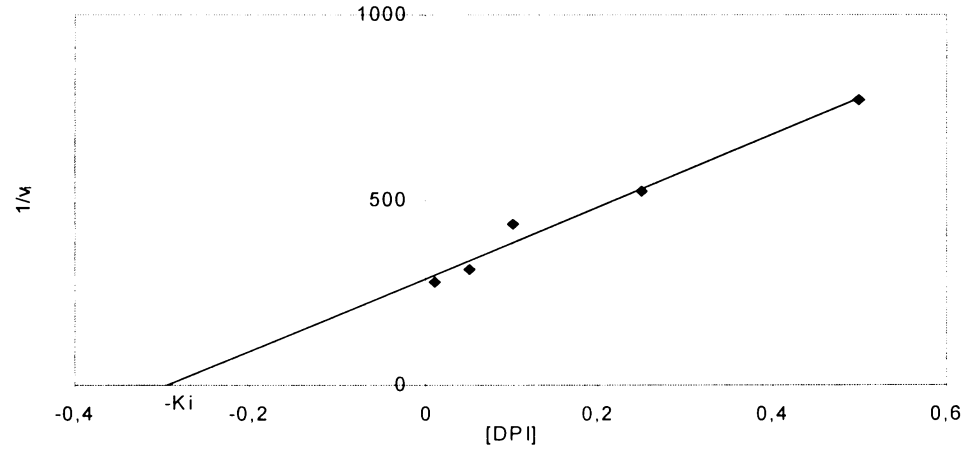

Fig. 39 *Dixon plot for reaction of LAD with AO in the presence of DPI. 1/v means reciprocal initial rate 1/[mmol.min^{-1}], [DPI] means concentration of inhibitor [mmol. l^{-1}] for this reaction. Ki is 0,29 mmol.l^{-1}*

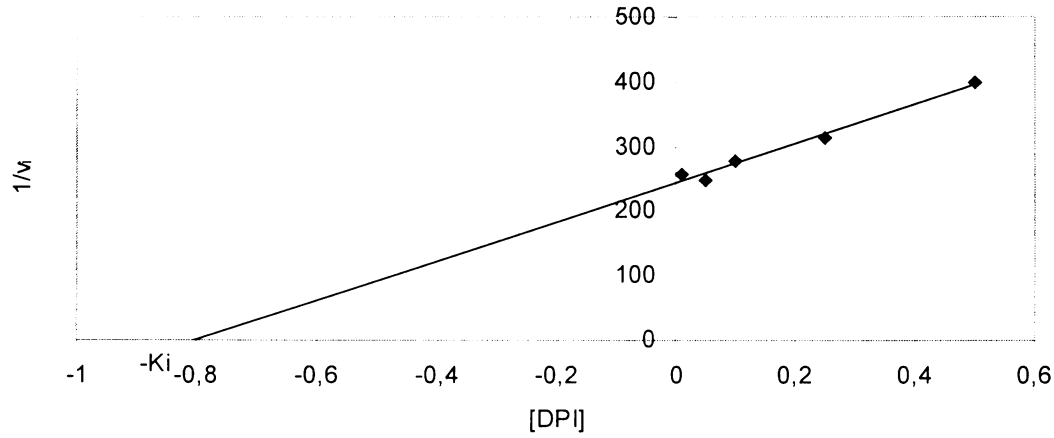

Fig. 40 *Dixon plot for reaction of LAD with SNAP in the presence of DPI. 1/v means reciprocal initial rate 1/[mmol.min^{-1}], [DPI] means concentration of inhibitor [mmol. l^{-1}] for this reaction. Ki is 0,80 mmol.l^{-1}.*

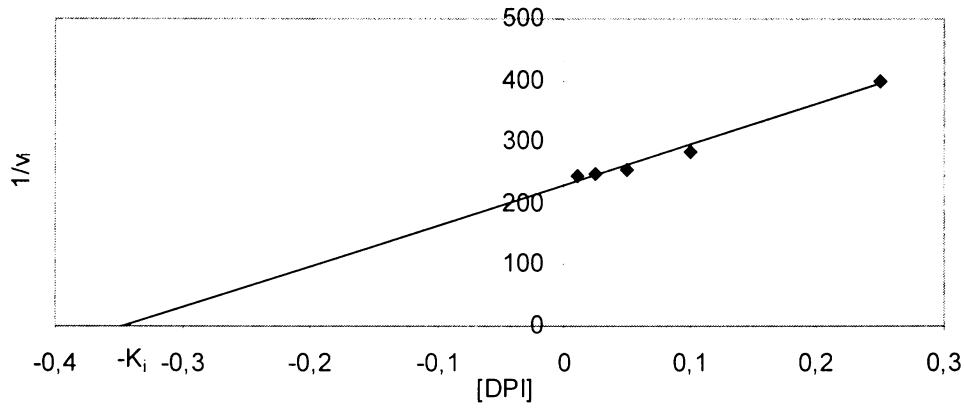

Fig. 41 *Dixon plot for reaction of LAD with SNOG in the presence of DPI. 1/v means reciprocal initial rate 1/[mmol.min^{-1}], [DPI] means concentration of inhibitor [mmol. l^{-1}] for this reaction. Ki is 0,35 mmol.l^{-1}*

6.4.3. Determination of Inhibition Constants

DPI inhibited all reaction with a high efficiency. DPI inhibited the reactions of all substrates and with both DP and LAD in a concentration-dependent manner achieving from 42 to 55% inhibition (Fig. 42 – 48). Calculated IC 50 values: DP/GTN: 0,2 mmol/l, DP/SNOG: 0,1 mmol/l, DP/SNAP: 3,2 mmol/l, DP/FAL: 0,1 mmol/l, DP/AO: 4,9 mmol/l, LAD/GTN: 1,9 mmol/l, LAD/SNOG: 2,1 mmol/l, LAD/SNAP: 8,8 mmol/l, LAD/FAL: 2,7 mmol/l, LAD/AO: 3,2 mmol/l.

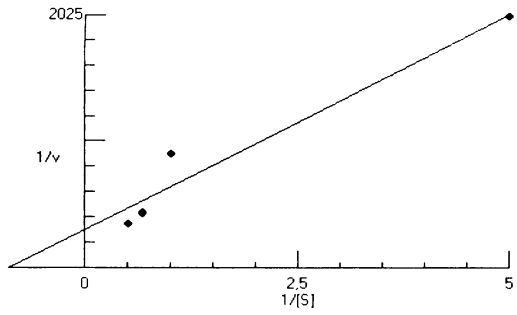

Fig. 42 *Lineweaver-Burk plot for reaction of DP with GTN in the presence of DPI. 1/v means reciprocal initial rate 1/[mmol.min^{-1}], 1/s means reciprocal concentration of substrate 1/[mmol. l^{-1}] for this reaction.*

Tab. 18 DP + GTN + DPI		
	Km (mmol)	Vmax (mmol.l^{-1} min^{-1})
Hanes plot results	3,88	0,0076
Lineweaver-Burk plot results	1,12	0,0033
Eadie-Hofstee plot results	0,93	0,0023
Hyperbolic regression results weighted	2,48 ± 1,68	0,03 ± 0,00

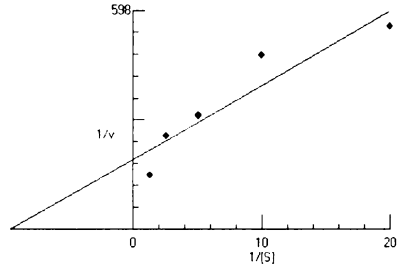

Fig. 43 *Lineweaver-Burk plot for reaction of DP with SNOG in the presence of DPI. 1/v means reciprocal initial rate 1/[mmol.min^{-1}], 1/s means reciprocal concentration of substrate 1/[mmol. l^{-1}] for this reaction.*

Tab. 19 DP + SNOG +DPI		
	Km (mmol)	Vmax (mmol.l^{-1} .min^{-1})
Hanes plot results	0,31	0,008696
Lineweaver-Burk plot results	0,11	0,005199
Eadie-Hofstee plot results	0,14	0,006101
Hyperbolic regression results weighted	0,17 ± 0,026	0,00623 ± 0,004935

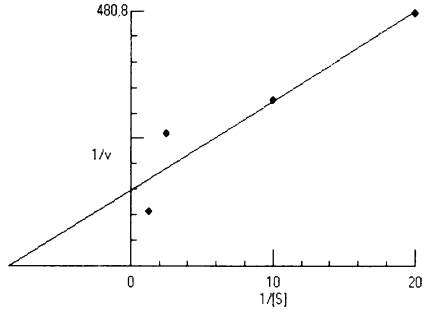

Fig. 44 *Lineweaver-Burk plot for reaction of LAD with SNOG in the presence of DPI. 1/v means reciprocal initial rate 1/[mmol.min^{-1}], 1/s means reciprocal concentration of substrate 1/[mmol. l^{-1}] for this reaction.*

Tab. 20 LAD + SNOG + DPI		
	Km (mmol)	Vmax (mmol.l^{-1} .min^{-1})
Hanes plot results	0,37	0,01185
Lineweaver-Burk plot results	0,12	0,00699
Eadie-Hofstee plot results	0,15	0,008342
Hyperbolic regression results weighted	0,10 ± 0,038	0,00626 ± 0,01023

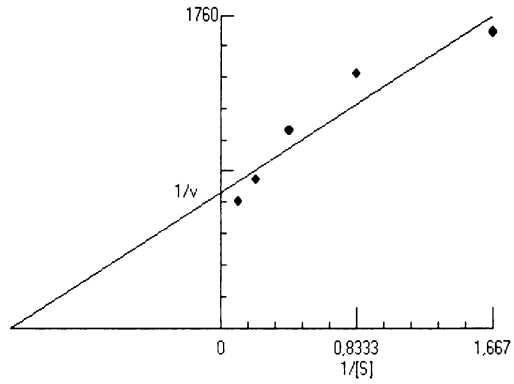

Fig. 45 *Lineweaver-Burk plot for reaction of DP with AO in the presence of DPI. 1/v means reciprocal initial rate 1/[mmol.min^{-1}], 1/s means reciprocal concentration of substrate 1/[mmol. l^{-1}] for this reaction.*

Tab. 21 DP + AO + DPI		
	Km (mmol)	Vmax (mmol.l^{-1} .min^{-1})
Hanes plot results	1,45	0,00159
Lineweaver-Burk plot results	0,78	0,001306
Eadie-Hofstee plot results	0,90	0,001383
Hyperbolic regression results weighted	1,01 ± 0,098	0,001412 ± 5,102e-04

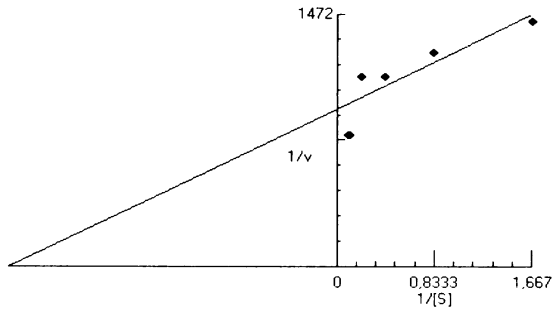

Fig. 46 *Lineweaver-Burk plot for reaction of LAD with AO in the presence of DP. 1/v means reciprocal initial rate 1/[mmol.min^{-1}], 1/s means reciprocal concentration of substrate 1/[mmol. l^{-1}] for this reaction.*

Tab. 22 LAD + AO + DPI		
	Km (mmol)	Vmax (mmol.l^{-1} .min^{-1})
Hanes plot results	1,13	0,001366
Lineweaver-Burk plot results	0,36	0,001087
Eadie-Hofstee plot results	0,41	0,001129
Hyperbolic regression results weighted	1,01 ± 0,098	0,001081 ± 3,787e-04

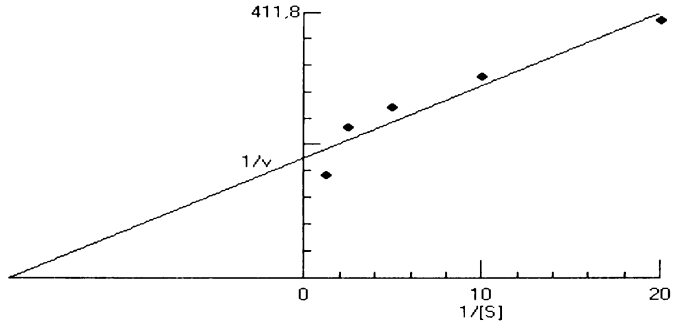

Fig. 47 *Lineweaver-Burk plot for reaction of DP with SNAP in the presence of DPI. 1/v means reciprocal initial rate 1/[mmol.min^{-1}], 1/s means reciprocal concentration of substrate 1/[mmol. l^{-1}] for this reaction.*

Tab. 23 DP + SNAP + DPI		
	Km (mmol)	Vmax (mmol.l^{-1} .min^{-1})
Hanes plot results	0,14	0,0070
Lineweaver-Burk plot results	0,06	0,0053
Eadie-Hofstee plot results	0,07	0,0057
Hyperbolic regression results weighted	0,07 ± 0,008	0,0068 ± 0,08037

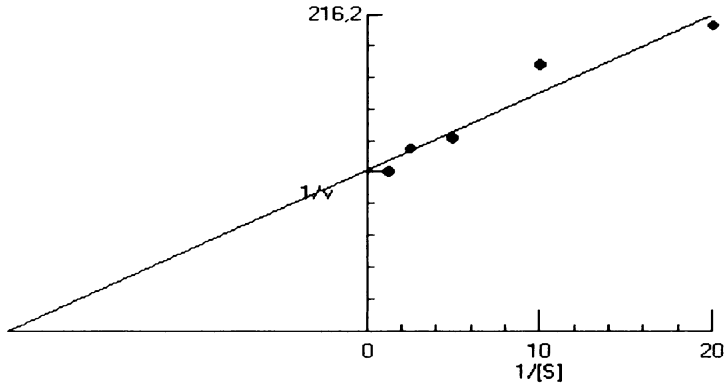

Fig. 48 *Lineweaver-Burk plot for reaction of LAD with SNAP in the presence of DPI. 1/v means reciprocal initial rate 1/[mmol.min^{-1}], 1/s means reciprocal concentration of substrate 1/[mmol. l^{-1}] for this reaction.*

Tab. 24 LAD + SNAP + DPI

	Km (mmol)	Vmax (mmol.l^{-1} .min^{-1})
Hanes plot results	0,07	0,00987
Lineweaver-Burk plot results	0,05	0,00910
Eadie-Hofstee plot results	0,05	0,00927
Hyperbolic regression results weighted	0,05 ± 0,004	0,00935 ± 0,00199

Effect of PH - the time-course of NP reaction with LAD or DP, resp. inhibited by PH has prevented the determination of kinetic parameters (Fig. 49, 51). PH significantly lowered the nitrite production up to 54 or 32%, resp. of control reaction (Fig. 50, 52)

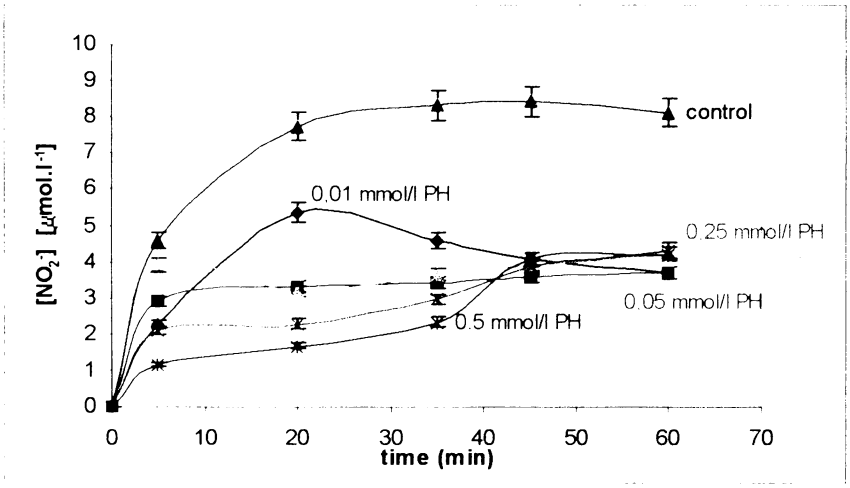

Fig. 49 *Effect of inhibitor PH on nitrite production in reaction of LAD with NP. Control is reaction without inhibitor. Results are expressed as mean ± SEM (n=3).*

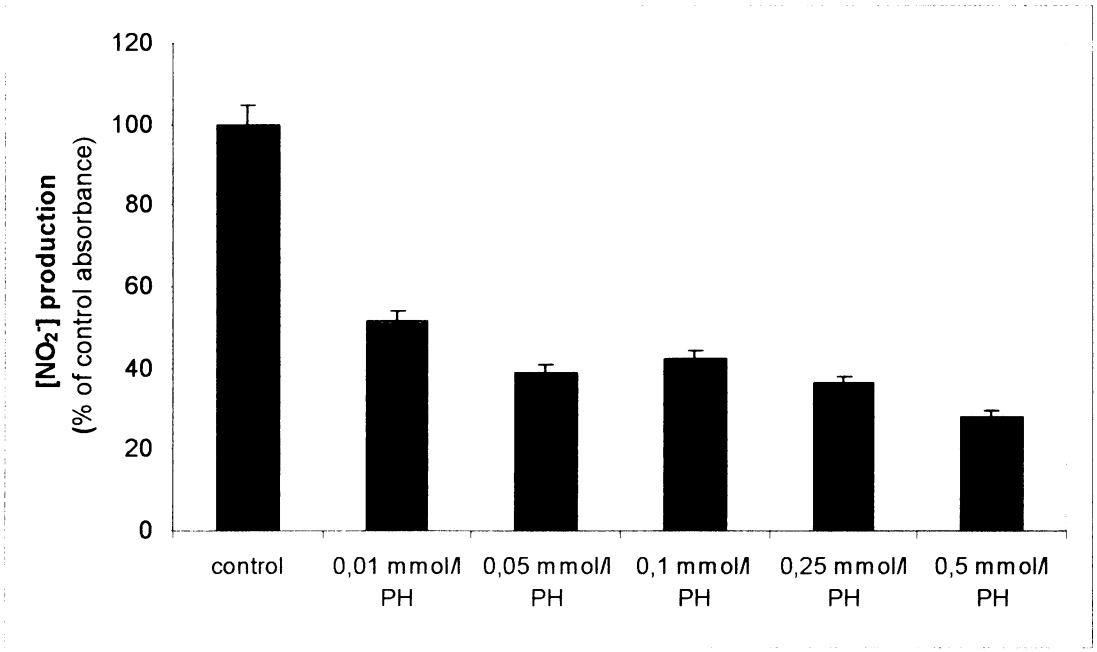

Fig. 50 *Inhibition of nitrite production in reaction of LAD with NP by PH. Control is reaction without inhibitor. Bars represents amount of nitrite at t=20 min and show the mean ± SEM (n=3).*

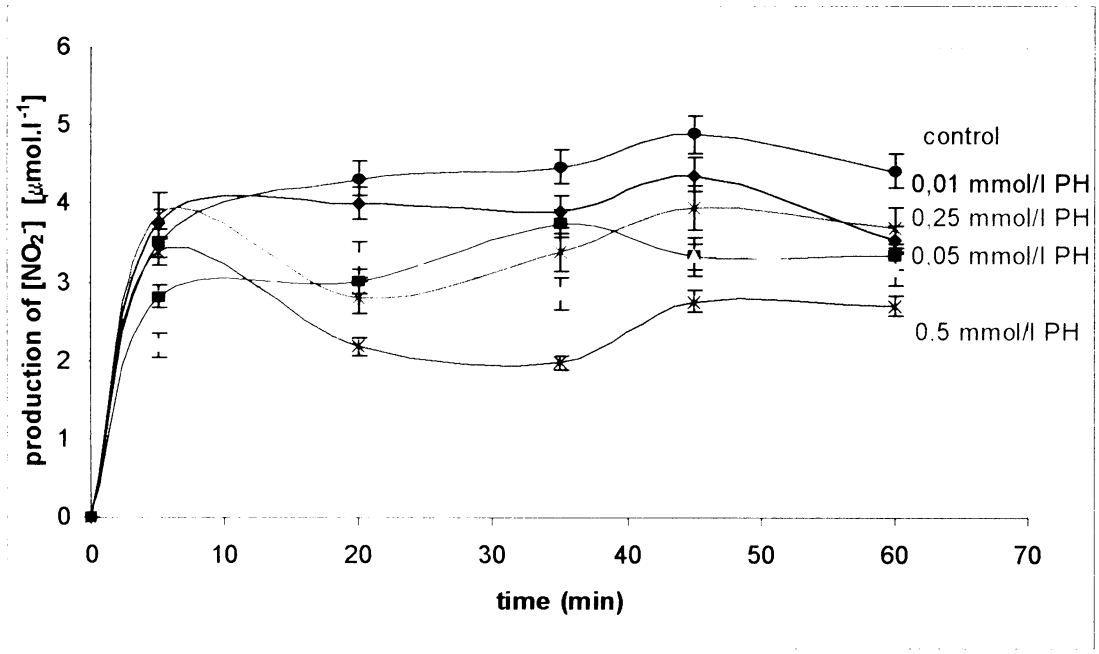

Fig. 51 *Effect of inhibitor **PH** on the reaction of DP with NP. Control is reaction without inhibitor. Results are expressed as mean ± SEM (n=3).*

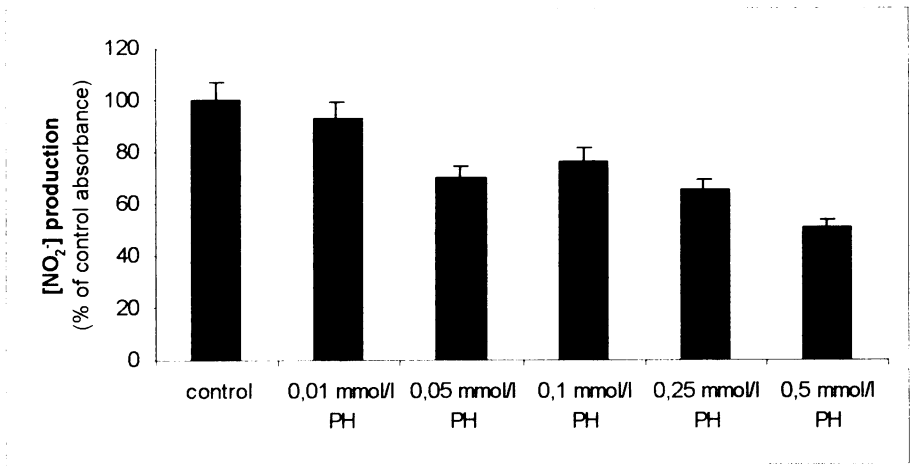

Fig. 52 *Inhibition of nitrite production in reaction of DP with NP by* ***PH****. Control is reaction without inhibitor. Bars represents amount of nitrite at t=20 min and show the mean ± SEM (n=3).*

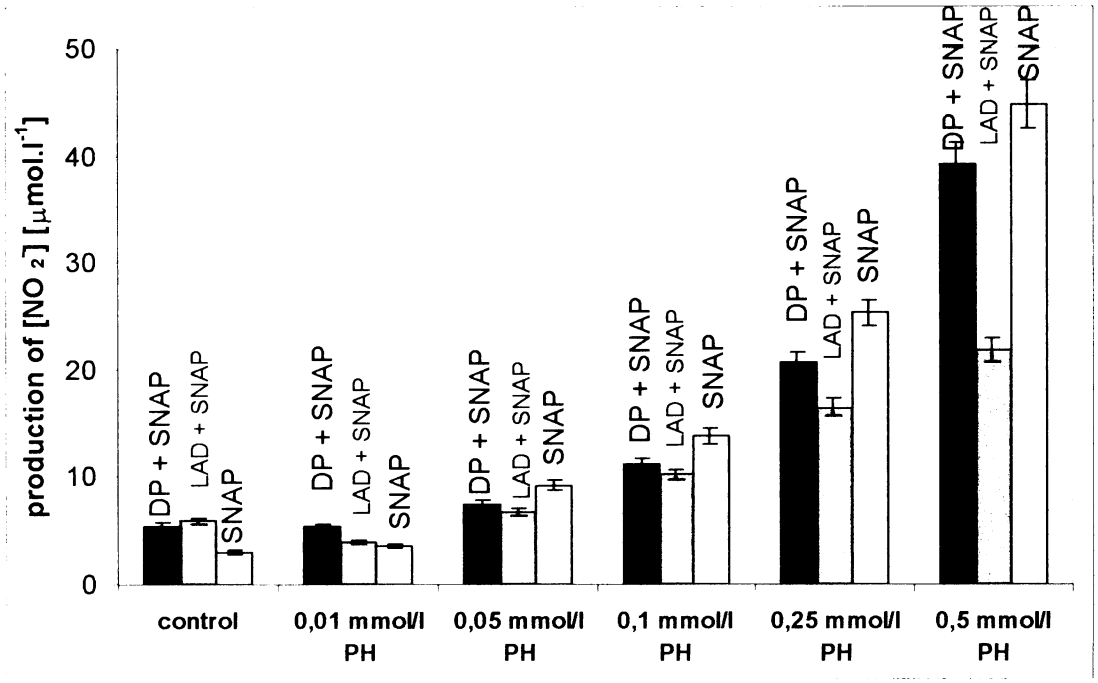

Fig. 53 *Effect of inhibitor* ***PH*** *on SNAP.*

Reaction with DP with SNAP or LAD and SNAP or SNAP alone are depicted. Control contained no inhibitor. Bars represents amount of nitrite at t=10 min and show the mean ± SEM (n=2).

Effect of ER

ER lowered the GTN transformation by LAD or DP, resp. with about 7-20% efficiency. The determination of parameters was abolished by high absorbance of ER.

When ER was added to DP and SNOG, the solution rapidly changed color from yellow to pale yellow and the total absorbance was higher than that of sample without ER. During the reaction, this yellow color turned gradually back. Change of color was proportional to concentration of ER in samples. Samples with low concentration of ER changed color faster than samples with high concentration. This kind of time-dependent inhibition was not observed by LAD, data not shown.

Effect of MC

MC inhibited the conversion of NP in a time-dependent manner. After 20 min, the inhibition of LAD reaction was about 62% for every concentration of MC. Than inhibition decreased in time, but the lowest concentration of MC inhibited the reaction with greater efficiency.

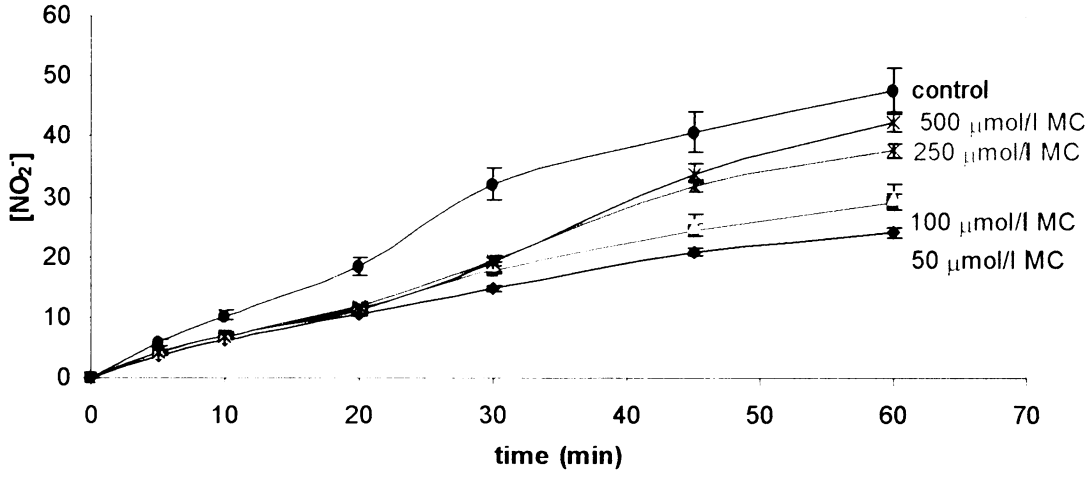

Fig. 54 *Effect of inhibitor **MC** on nitrite production in reaction of LAD with NP. Control means reaction without inhibitor. Results are expressed as mean ± SEM (n=3).*

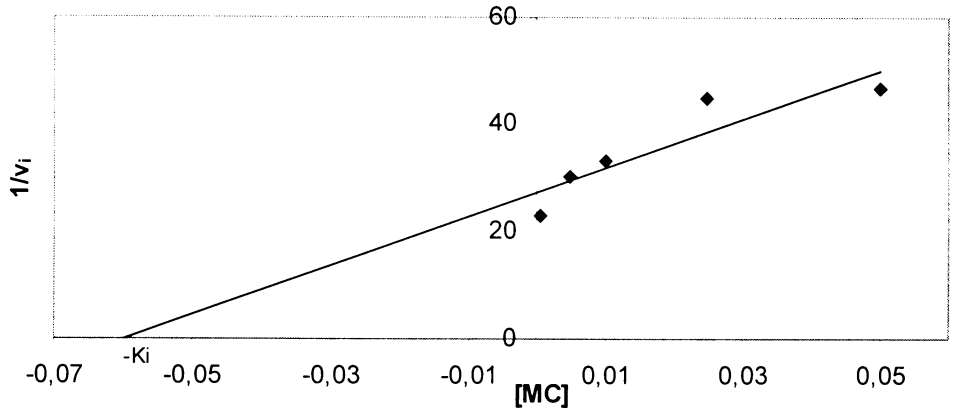

Fig. 55 *Dixon plot for reaction of LAD with NP in the presence of MC. 1/v means reciprocal initial rate of inhibited reaction 1/[mmol.min^{-1}], [MC] means concentration of inhibitor [mmol. l^{-1}] for this reaction. K_i is 0,06 mmol.l^{-1}.*

Tab. 25 *Overview of inhibitory effect of inhibitors: + means inhibition, -means no inhibitory effect, +/- indicates reactions, which were inhibited with low efficiency, or markedly influenced.*

	DPI		PA		ER		PH		L-NAME		MC	
	DP	LAD	DP	LAD	DP	LAD	DP	LAD	DP	LAD	DP	LAD
GTN	+	+	–	–	+/-	+/-	–	–	–	–	–	–
NP	+	+	–	–	–	–	+	+	–	–	+/-	+/-
AO	+	+	–	–	–	–	–	–	–	–	–	–
SNAP	+	+	–	–	–	–	+/-	+/-	–	–	–	–
SNOG	+	+	–	–	–	+/-	–	–	–	–	–	–
FAL	+	+	–	–	–	–	–	–	–	–	–	–

6.4.4. Oxidase activity

A pronounced decrease of absorbance in time was observed by both enzymes, suggesting a NADH oxidation.

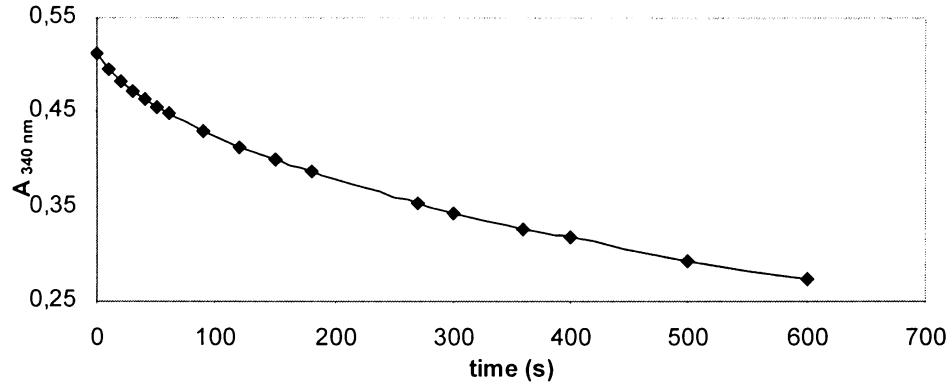

Fig. 56 *Effect of oxidase activity of LAD. Decrease of absorbance at 340 nm represents decrease of NADH concentration in the presence of LAD .*

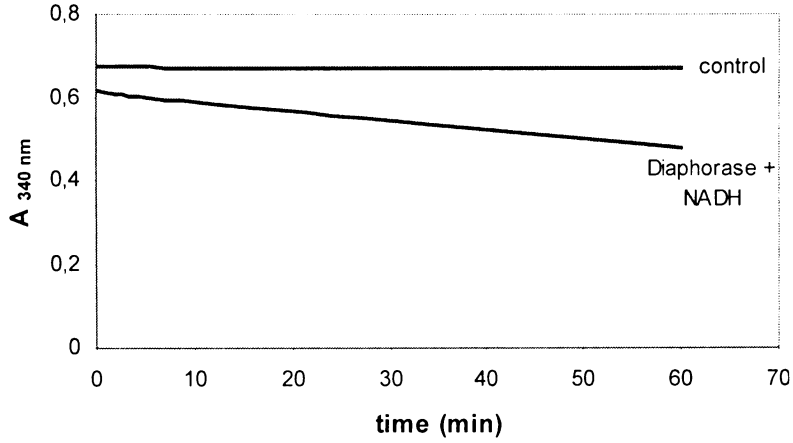

Fig. 57 *Effect of oxidase activity of DP. Increase of absorbance at 340 nm represents increase of NADH concentration in the presence of DP or without (control).*

6.4.5. Nitrosylation reaction of SNAP and SNOG

In the presence of BSA, samples with SNAP or SNOG showed lower amounts of nitrites than control without BSA. The effect of BSA is much pronounced by SNAP than by SNOG.

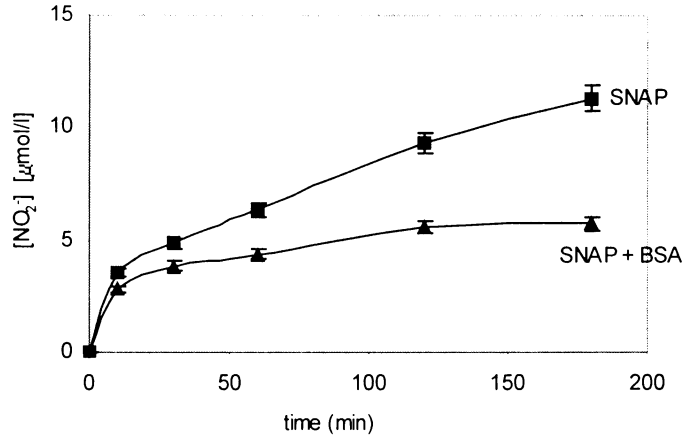

Fig. 58 *SNAP-mediated nitrosylation of BSA..*

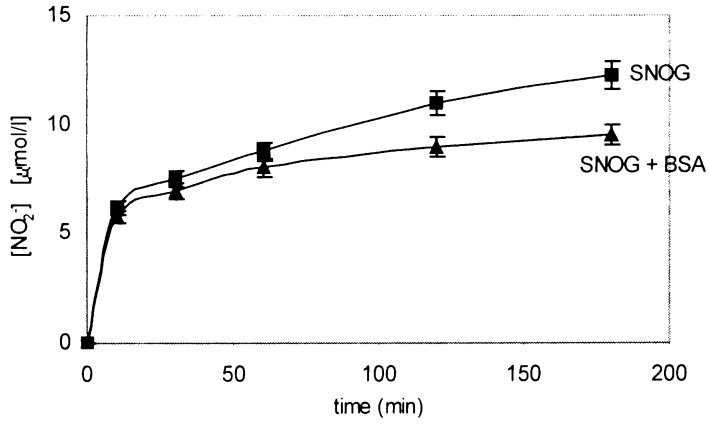

Fig. 59 *SNOG -mediated nitrosylation of BSA.*

7. DISCUSSION

Discussion

Previous studies in our laboratory [II, III] have reported conversion of GTN and FAL by diaphorase from *Clostridium kluyveri* to nitrites and nitrates with nitric oxide as an intermediate.

The present study not only confirmed the earlier findings but also tested another five different vasodilating compounds beside GTN and FAL. This study showed that analogous enzyme in mammals LAD is also able to transform these substances in similar way. Previously, tests undertaken in our laboratory have shown that a very sensitive fluorescence method is not applicable for this model of enzymatic transformation, because the presence of NADH significantly influence the assay. The Griess assay was found as the most suitable method to quantify endproduct of NO-donors transformation (nitric oxide and /or other products leading to nitrites).

With the exception of FAM, selected compounds (GTN, SNAP, SNOG, NP, AO and FAL) were converted by both DP and LAD in the presence of NADH.

Kinetic parameters (V_{max} and K_m) were determined for all reactions except of NP. This parameters are different from previously published results for reaction of DP with GTN or FAL, resp. [II]. However our results are calculated according to initial rate of reaction.

An experiment with BSA showed, that SNAP and SNOG, beside spontaneous decomposition, are able to nitrosylate thiols in proteins, what leads to decrease of nitrite levels in samples. However, concentration of BSA was significantly higher than that of LAD or DP, resp. in all assays.

It should be pointed out that many earlier studies [e.g.133] showed that DP has no NADH oxidase activity. In the present study, time-dependent decrease of NADH concentration was measured photometrically at 340 nm under different experimental conditions. The reason for the discrepancy observed between the studies is not known. It is unclear, whether this decrease of NADH concentration is NADH oxidase activity. Experiments were carried out under aerobic conditions, what may account for the observed difference and needs further investigation. Decrease of NADH was taken in consideration in all experiments.

DPI inhibited the reactions of all substrates and with both DP and LAD in a concentration-dependent manner achieving inhibition from 42 to 55%. The kinetic and inhibition constants were determined. In spite of the fact that DPI inhibits one electron reactions of flavin cofactors, it could be concluded, that all compounds converted by DP and LAD are

transformed via one electron reduction or oxidation. This presumption agrees with reported studies of DPI-inhibited biotransformation of organic nitrates in rat aortic ring and *in vivo* experiments.

PH had no effect on the diaphorase activity in the whole set (GTN, FAL, AO, SNAP and SNOG) except of the conversion of NP by DP, resp. LAD in the presence of NADH. PH lowered the diaphorase activity to about 50%, resp. 28% of the control value, but the time-course of reaction has prevented the determination of inhibition type or constants. PH significantly increased the decomposition of SNAP in the presence of NADH. This reaction was not influenced by DP or LAD.

ER inhibited the conversion of GTN by LAD or DP, resp. with about 7-20% efficiency. Interestingly, high concentrations of ER did not inhibit the conversion of SNAP. Absorbance values were even higher than control without inhibitor . In contrast, low concentrations of ER inhibited the reaction for 25 min with 100% efficiency, than inhibition decreased, but at the same time, the highest concentration of ER began to inhibit the reaction with about 45% efficiency.

ER influenced the absorbance of samples with DP and SNOG. The observed change of color of samples from yellow to pale yellow could be attributed to reduction of flavin. Further, ER may serve as a substrate in reaction of DP with SNOG.

L-NAME (NOS inhibitor) and PA (NOS and cytochrom P450 inhibitor) have not abolished the transformation of substrates by DP or LAD. The inhibitor of reductases DC, was excluded from inhibition assays, because of absorption maxima near measured absorbance of Griess reaction.

The transformation of NO-donors by LAD has not been described so far. Due to similarity of LAD from porcine heart used in these experiments to the human LAD, especially conversion of GTN by this mammal enzyme is important, because GTN is widely used as a potent drug.

8. CONCLUSION

Conclusion

The diaphorase and dihydrolipoamide dehydrogenase were found to be involved in biotransformation of different types of vasorelaxing compounds.

With the exception of formamidoxime, selected vasorelaxing compounds (glycerol trnitrate, nitroprusside, S-nitroso- *N*-acetylpenicilamine, S-nitrosoglutathione, formaldoxime and acetaldoxime) were converted to nitrites via NO by diaphorase and dihydrolipoamide dehydrogenase. For these reactions, kinetic parameters (V_{max} and K_m) were obtained.
Nitroprousside was found to be transformed by both enzymes, but the complicated time-course of reaction did not allow to determine kinetic parameters.
The diaphorase-mediated conversion to nitric oxide was more efficient than that of dihydrolipoamide dehydrogenase.

Inhibition studies showed that diphenyleneiodonium inhibited all reactions. Ethoxyresorufin inhibited the reaction of dihydrolipoamide dehydrogenase with glycerol trinitrate. Phenidone, miconazole and ethoxyresorufin inhibited or affected the reactions of nitroprousside with both enzymes. L-NAME and proadifen had no effect on conversion of selected substances. Inhibitor dicoumarol was excluded, because of interference with the used detection method.
Inhibition constants and types of inhibition were determined for some reactions.

In addition, a decrease of NADH level by both enzymes in the absence of substrates was observed, suggesting a NADH oxidase activity.

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Svoluji k zapůjčení této diplomové práce pro studijní účely a prosím, aby byla vedena evidence vypůjčovatelů.

Jméno a příjmení s adresou	Číslo OP	Datum vypůjčení	Poznámka