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Organic Nitrate Esters: Clinical Use and Mechanisms of Actions*

JOHAN AHLNER,[a]† ROLF G. G. ANDERSSON,[b] KRISTINA TORFGÅRD,[a] AND KRISTER L. AXELSSON[b]

[a]*Department of Clinical Pharmacology and* [b]*Department of Pharmacology, Faculty of Health Sciences, University Hospital, Linköping, Sweden*

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†Correspondence: Department of Clinical Pharmacology, Faculty of Health Sciences, University Hospital, S-581 85, Linköping, Sweden.

I. Introduction

GTN‡ was first synthesised in 1846 by the Italian chemist Ascanio Sobrero, and the first literature report describing the synthetic procedure was given 1 year later (Sobrero, 1847). The possible value of this new compound as a therapeutic agent soon attracted the attention of the homoeopathic physician Constantin Hering, and in the decades to follow it was used as a remedy for a number of diseases in homoeopathic medicine. In 1853 Hering suggested that GTN could be of therapeutic value in the treatment of angina pectoris. The first description of GTN as a therapeutic agent for the treatment of angina pectoris appeared in 1879 in the classical articles in the *Lancet* by the English physician William Murrel. Since then it has remained an important drug in the treatment of angina pectoris. However, despite an enormous amount of clinical experience, there are still controversies regarding the proper use of GTN in clinical practice. Subsequent to the introduction of GTN as a therapeutic agent for the treatment of angina pectoris, other nitro compounds with similar chemical properties as GTN have been introduced. These include ISDN, IS-5-MN, and pentaerythritol tetranitrate. In addition to these, there also are other compounds such as molsidomine and amyl nitrite that show a pharmacodynamic resemblance to the aforementioned drugs.

The invention of GTN and its use as a powerful explosive soon attracted the attention of Alfred Nobel, who mixed GTN with silica guhr to form a product that was named dynamite. The use of GTN as the active agent in the production of explosives was later supplemented with other compounds, of which EGDN is of particular interest because it shows a striking similarity to GTN both in its chemical properties and biological effects.

All compounds used as vasodilators in the treatment of angina pectoris have a similar molecular structure with the nitrate ester bond (R—O—NO_2) as an essential feature; this chemical group lends unique biological properties to this group of compounds. The nitrate ester group is also important structurally because it distinguishes the organic nitrate esters from nitro compounds, which possess a carbon-nitrogen bond (R—C—NO_2), and nitric oxide-containing compounds. The latter group includes the well-known vasodilator SNP, and the nitro compounds include various explosives such as trinitro toluene and the fuel additive nitromethane. The structural formulas for some members of these different groups of drugs are given in figure 1. The term "nitrate" vasodilators has often been used to identify vasodilator compounds belonging to any of these groups. However, to clearly stress the important chemical and functional differences between nitrate esters and nitro- or nitric oxide-containing drugs, we believe that the term "organic nitrate esters" should be adopted and used exclusively for drugs containing the —O—NO_2 moiety. Organic nitrate esters are polyol esters of nitric acid, whereas the chemically related group of substances called organic nitrite esters are polyol esters of nitrous acid. Amyl nitrite (fig. 1) can be considered representative for the organic nitrite esters.

Because of the similarities in chemical structure, all organic nitrate esters share similar pharmacological properties, including basic mechanism of action. However, it seems that mechanistic differences do exist

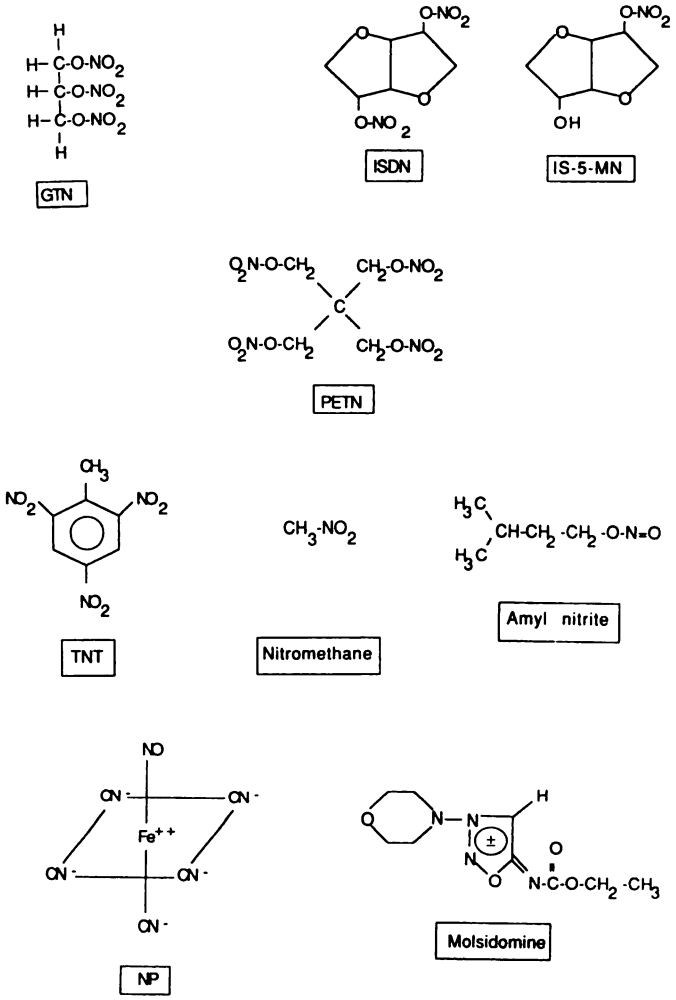

FIG. 1. Chemical structure of selected organic nitrate esters. The structure of some pharmacologically or structurally related compounds is shown for comparison. PETN, pentaerythreitol tetranitrate; NP, nitroprusside; TNT, trinitro toluene.

‡Abbreviations: GTN, glyceryl trinitrate, nitroglycerin; EGDN, ethylene glycol dinitrate; ISDN, isosorbide dinitrate; IS-5-MN, isosorbide-5-mononitrate; NA, noradrenaline; SNP, sodium nitroprusside; cGMP, cyclic guanosine-3′,5′-monophosphate; cAMP, cyclic adenosine-3′,5′-monophosphate; ANP, atrial natriuretic peptide; cG-Pk, cyclic GMP-dependent protein kinase; ADP, adenosine diphosphate; EDRF, endothelium-derived relaxing factor; EC_{50}, concentration of a drug at half-maximal effect; IP_3, inositol 1,4,5-trisphosphate; ATP, adenosine tri phosphate; SH, sulfhydryl; GDN, glyceryl dinitrate; PVC, polyvinyl chloride; GC, gas chromatography; ECD, electron capture detection; AUC, area under curve; IS-2-MN, isosorbide-2-mononitrate; APTT, activated partial thromboplastin time; ECG, electrocardiogram; PCWP, pulmonary capillary wedge pressure; PTCA, percutaneous transluminal coronary angioplasty.

among the various members of this group of compounds, although the functional importance of this finding is not yet clear. Differences in chemical structure also have obvious consequences for the pharmacokinetic behaviour of the organic nitrate esters, an observation that is of great clinical importance. There is also the general problem of handling lipophilic agents, such as organic nitrate esters. Because of their physical and chemical properties, interactions may occur between the pharmacological agent and the materials of the experimental apparatus or, in clinical studies, with the infusion sets; these problems are discussed in the following sections.

The present review is an attempt to summarise the present knowledge (as of autumn 1990) concerning some important basic and clinical aspects of organic nitrate ester pharmacology.

II. In Vitro Evaluation of the Effects of Nitrate Esters

A variety of nitrites and nitrates are useful in medicine because of their relaxing effect on various smooth muscles. The smooth muscle-relaxing effect of GTN was recognised soon after its introduction as a homeopathic remedy (Fye, 1986). The therapeutic effect of GTN in angina pectoris and congestive heart failure is mainly due to the relaxant effect the drug has on vascular smooth muscle. Dilation of venous capacitance and arterial resistance vessels, as well as dilation of coronary arteries, is of importance (Hollenberg and Go, 1984; Corwin and Reiffel, 1985).

A. In Vitro Experimental Conditions during Studies of Vasodilation Induced by Nitrate Esters

Sensitivity to the relaxant action of nitrate esters, e.g., GTN, varies considerably among different types of smooth muscle preparations as well as on the experimental conditions used. Most in vitro studies have demonstrated that therapeutically relevant concentrations of GTN have only a slight relaxant effect on peripheral arteries (Mackenzie and Parratt, 1977; Axelsson et al., 1979; Weinheimer et al., 1983; Ahlner et al., 1985; Torresi et al., 1985) and veins (Mackenzie and Parrat, 1977; Nakajima and Nosaka, 1983; Toyoda et al., 1986).

Several potential explanations exist for the discrepancy between the clinically effective concentration of GTN and the concentration found to be effective in vitro (Ahlner et al., 1987a,b). Possibly of importance in in vitro studies is adsorption of GTN to the experimental equipment; this has been examined by Ahlner et al. (1987a,b,c), who found that GTN was retained by the manufactured materials (plexiglass and glass) of organ baths. To minimise potential interference from adsorption, use of equipment constructed form inert material, such as polyethylene, has been recommended (Ahlner, 1987; Ahlner et al., 1987a,b).

Another problem caused by adsorption of GTN to organ baths is that the drug can be liberated during reuse of the equipment. Even very careful washing with ethanol does not remove all traces of GTN from organ baths, and the nitro compound can be reliberated during preequilibration of in vitro preparations, making them partly tolerant and thus causing substantial changes in EC_{50} values (Ahlner et al., 1987a,b).

Previous in vitro studies have demonstrated marked differences in IC_{50} values for GTN-induced relaxation in different vascular preparations (table 1); the values vary between 2 and 14,800 nM. Measures taken to prevent potential problems caused by the reliberation of GTN from equipment resulted in a dramatic increase in sensitivity to the drug in a peripheral artery preparation in vitro (Ahlner et al., 1987a,b). When disposable polyethylene vials were used in concentration-effect experiments, picomolar amounts of GTN caused relaxation in strips of bovine mesenteric arteries. The concentration-response curves obtained in the polyethylene vials had a biphasic pattern, with a pD_2 value of 11.9 for the high affinity component and a pD_2 of 7.5 for the low affinity component (fig. 2; Ahlner et al., 1987a). The high affinity part of the concentration-response curve fell within the concentration range of the therapeutic plasma concentrations (for example, see Heidemann et al., 1987). GTN has previously been shown to cause a biphasic relaxation pattern in canine coronary artery (Kamitani, 1984) and rabbit femoral and mesenteric artery (Toyoda et al., 1986; Rösen et al., 1987). Moreover, Malta (1989) recently showed biphasic relaxant curves to GTN in rat aortic preparations. The sensitivity to GTN shown by dog, rabbit, and rat arteries was, however, far less pronounced (100- to 1000-fold) than in bovine mesenteric arteries.

In both arterial and venous preparations, GTN caused a biphasic relaxation pattern, and the threshold for relaxation indicated a higher sensitivity to GTN of veins than of arteries (Rösen et al., 1987). The biphasic relaxant pattern of GTN seemed to be specific to this compound, because other nitro compounds, such as ISDN, IS-5-MN (Toyoda et al., 1986; Axelsson et al, 1989b; Torfgård et al., 1990a), EGDN, or butanol-1,2,3,4-tetranitrate (fig. 2), showed no biphasic dose-response pattern. Because the latter two organic nitrates are closely related structurally to GTN, it is apparent that GTN is unique in displaying a biphasic concentration-effect curve.

In addition to the equipment, the contractile stimuli used to induce tone is also of importance for the in vitro determination of IC_{50} values for GTN-induced vascular relaxation. GTN is a more potent relaxing agent in vessels contracted by NA, phenylephrine, 5-hydroxytryptamine (serotonin), and histamine than in vessels contracted by a high (80 to 127 mM) concentration of K^+ (Mikkelsen et al., 1978; Axelsson et al., 1979; Ahlner et al., 1985; Karaki et al., 1984; Mackenzie and Parratt, 1977); this applies to both venous (Mikkelsen et al., 1978; Ahlner et al., 1985; Mackenzie and Parratt, 1977) and arterial smooth muscle (Axelsson et al., 1979; Karaki et

TABLE 1

EC_{50} values obtained in studies in vitro of the relaxant effect of GTN on blood vessels from different species and different anatomical regions

Vessel	EC_{50} (nM)	Constractile stimuli	Reference
Bovine coronary artery	200	5-Hydroxytryptamine	Ahlner et al., 1985
Bovine mesenteric artery	27	Histamine	Axelsson et al., 1979
Bovine mesenteric vein	1,000	5-Hydroxytryptamine	Axelsson, 1984
Bovine pulmonary artery	40	K^+	Edwards et al., 1984
Bovine intrapulmonary vein	2	K^+	Edwards et al., 1984
Canine coronary artery	256	K^+	Kamitani, 1984
Canine coronary artery	2,400	K^+	Shibata et al., 1984
Canine coronary artery	10	NA	Wennheimer et al., 1983
Canine mesenteric artery	64	Thromboxane A_2	Miwa and Toda, 1985
Canine mesenteric artery	7,900	K^+	Shibata et al., 1984
Canine femoral artery	14,800	K^+	Shibata et al., 1984
Canine femoral artery	5,000	NA	MacKenzie and Parrat, 1977
Canine femoral vein	610	K^+	Shibata et al., 1984
Rabbit femoral artery	1,622	NA	Toyoda et al., 1986
Rabbit femoral artery	1,000	NA	Nakajima and Nosaka, 1983
Rabbit femoral vein	5	NA	Nakajima and Nosaka, 1983

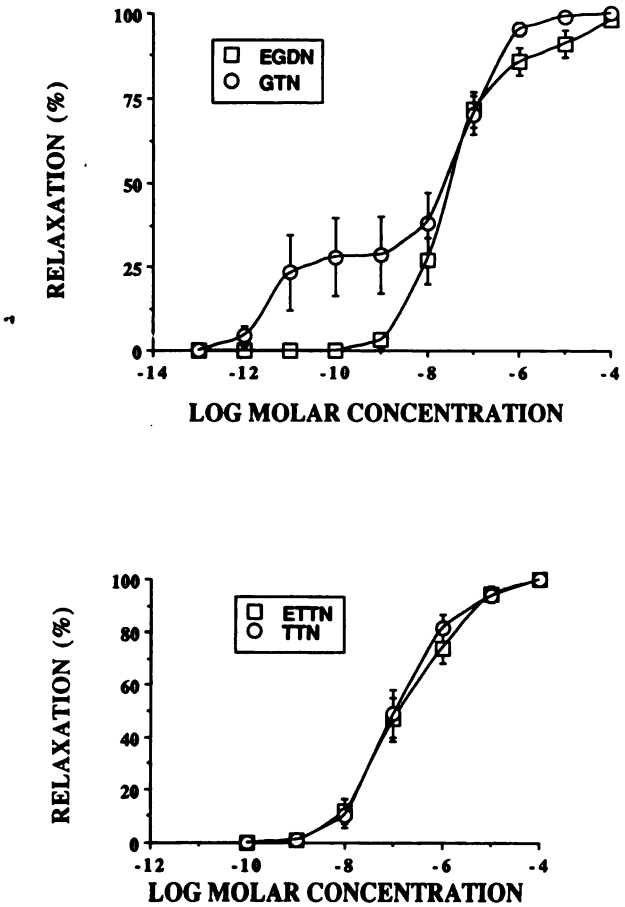

FIG. 2. Top, Concentration-effect curves for GTN and EGDN in bovine mesenteric arteries contracted with 3 mM phenylephrine. Bottom, Concentration-effect curves for 1,2,3,4-tetranitratobutan (erythritol tetratnitrate; ETTN) and racemic threitol tetranitrate (TTN) in bovine mesenteric arteries contracted with 3 mM phenylephrine. Means ± SEM; $n = 5$ to 7.

al., 1984; Mackenzie and Parratt, 1977). The same situation has been found for SNP (Hester et al., 1979; Karaki et al., 1980).

The concentration of the agent used to contract the vessels also seems to influence the relaxant effect of GTN. Karaki et al. (1984) and Yanagisawa et al. (1989) found an inverse relationship between the concentration of NA or potassium used to contract arterial tissues and the relaxant effect of GTN; a similar observation has been made using SNP in canine renal arteries contracted by NA or potassium (Karaki et al., 1980). However, differences might exist between species and/or vascular beds in this respect. Shibata et al. (1984) compared the inhibitory effect of GTN on vascular smooth muscle contraction induced by potassium (10 to 70 mM) and NA (10 nM to 10 μM) in rabbit aorta, rabbit basilary artery, and cat coronary artery. In rabbit aorta, GTN was practically unable to relax potassium-induced contraction, whereas in rabbit basilary artery and cat coronary artery, there was an inverse relationship between the concentrations used of both potassium and NA and the vessel relaxation. In fact, in cat coronary arteries, the relaxation induced by GTN in potassium-contracted (10 to 40 mM) vessels was the same as that in vessels contracted by NA.

B. Regional and Species Differences in the Relaxant Effect of Nitrate Esters

The type of tissue (species, localisation, vein, or artery) used in in vitro studies of nitrate esters significantly affects the IC_{50} values for relaxation.

Regional differences, i.e., different relaxant effects of GTN on vessels from different anatomical regions in the same species and contracted by the same agent, have been studied by Shibata et al. (1986). These researchers investigated the relaxant effect of GTN on coronary artery, mesenteric artery, renal artery, and femoral vein from mongrel dogs; all studied vessels were contracted when potassium depolarisation (30 nM) was used. The femoral vein was found to be most sensitive to GTN, coronary arteries were somewhat more sensitive than mesenteric arteries, and renal arteries were least sensitive. Gharaibeh and Gross (1984) investigated the relax-

ant effect of GTN in large artery rings obtained from the coronary, femoral, mesenteric, and renal vascular beds of the dog. These authors found that GTN was more potent (10- to 10,000-fold) in relaxing contractions produced by NA in the coronary artery than in the other three vascular beds. Miwa and Toda (1985) compared the relaxant effect of GTN on canine mesenteric arteries and canine coronary arteries contracted by carboxylic thromboxane A_2. They found that coronary arteries were significantly more responsive to GTN than were mesenteric arteries.

In addition to differences among various anatomical regions, there seem to be differences in vessel responsiveness related to the sizes of the vessels within single regions. Harder et al. (1979) examined the effect of GTN on action potentials of large and small coronary arteries of dogs. They found the large arteries to be more sensitive to GTN than the small ones. This also was demonstrated by Tillmanns et al. (1979), who found that larger coronary arterioles were more responsive to GTN than smaller ones.

It has long been known that nitrate esters cause arterial and venous vasodilation. Several observations have indicated that there is a fundamental difference in the venous and arterial sides of the circulatory system concerning concentration-effect relationships of nitrate esters. In general, in vitro investigations have shown nitro compounds to be more effective as relaxant agents for venous tissue than for arterial tissue (Mackenzie and Parrat, 1977; Nakajima and Nosaka, 1983; Toyoda et al., 1986; Rösen et al., 1987). The nitro compounds caused dose-dependent relaxation of both arterial and venous preparations; the potency order was GTN > ISDN > IS-5-MN. The maximum responses to the three drugs and their IC_{50} values had a tendency to be larger for veins than for arteries (Toyoda et al., 1986). The threshold concentration for arterial and venous dilation differed approximately 10-fold, and the IC_{50} values differed by 10- to 100-fold (Nakajima and Nosaka, 1983; Rösen et al., 1987). One explanation for the arterial-venous difference in sensitivity was investigated by Toyoda et al. (1987); they demonstrated that GTN, ISDN, and IS-5-MN inhibited Ca^{2+} release from Ca^{2+} stores more effectively in venous than in arterial preparations. Moreover, Ito et al. (1978) showed that the relative reduction in membrane resistance during application of the same concentration of a nitro compound was less in the pulmonary artery than in the portal vein.

In vitro studies, in which vascular preparations from rabbits and dogs were used, demonstrated a higher sensitivity of veins compared with arteries to organic nitrate esters (Mackenzie and Parrat, 1977; Nakajima and Nosaka, 1983; Toyoda et al., 1986; Rösen et al., 1987). This is in contrast to the findings in our laboratory, in which bovine mesenteric arteries were found to be more sensitive to relaxation by organic nitrate esters than veins from the corresponding region (Axelsson, 1984; Bornfeldt and Axelsson, 1987). There may, thus, exist important species and regional differences regarding the relative sensitivity of arteries and veins to organic nitrate esters.

The experiments performed on dog and rabbit vascular preparations are consistent with results from in vivo studies of humans. For example, Imhof et al. (1980) demonstrated that human venous capacitance vessels are more sensitive than arterial vessels to low doses of GTN.

C. Differences in Vasodilatory Effects of Different Nitrate Esters

The organic nitrate esters GTN, ISDN, and IS-5-MN have been reported to have the same principal therapeutic and hemodynamic effects (Abrams 1985, 1987; Abshagen, 1987). The mechanism of action on the cellular and subcellular levels is considered to be partly similar (for details see section III).

Stiefel and Kreye (1984) compared the vasorelaxant effects of GTN, ISDN, and IS-5-MN on isolated renal arteries and veins from the rabbit. These authors found that all of the substances relaxed the veins at lower concentrations than was needed to relax the arteries; moreover, they found that ISDN and IS-5-MN were much more venoselective than was GTN. Similar observations were reported by Toyoda et al. (1986) using rabbit femoral artery and vein. Bassenge et al. (1981) and Bassenge and Strein (1986) studied the effect of GTN and IS-5-MN on dogs. In both studies, a 10-fold higher dose was needed to induce dilation on the arterial side. However, there is no firm evidence from clinical studies that selectivity for different vascular beds varies for GTN, ISDN, and IS-5-MN (Schneider et al., 1986).

Kamitani (1984), Toyoda et al. (1986), Rösen et al. (1987), and Ahlner et al. (1987a,b) have shown that GTN induces a biphasic pattern in vascular smooth muscle relaxation; this is suggestive of at least two partially different mechanisms of action. No similar evidence exists in the literature for a dual mechanism of the ISDN- or IS-5-MN-induced relaxation of vascular smooth muscle. Toyoda et al. (1986) compared the effects of GTN, ISDN, and IS-5-MN on femoral arteries and femoral veins from rabbits; a biphasic relaxation pattern was observed when arteries were exposed to GTN but not when they were exposed to the other two substances. Furthermore, the nitro compound, 2-nicotinamidoethyl nitrate (nicorandil), acting on various types of vascular beds, did not induce a biphasic relaxant pattern (Nabata and Sakai, 1983; Inoue et al., 1984; Shibata et al., 1986; Murakami et al., 1987). The possible dissimilarities between the individual organic nitrate esters, as far as the mechanism of action on the cellular level is concerned, will be discussed in section III.

The vascular effects of nitrate esters are of the utmost importance in the treatment of angina pectoris and congestive heart failure; effects on other target cells have

also been demonstrated. For example, a number of studies have shown an in vitro inhibition of platelet aggregation by nitrate esters (Schaffer et al., 1980; Ahlner et al., 1985; Loscalzo, 1985; De Caterina et al., 1988) and effects of GTN on myocardial metabolism. These effects of nitrate esters are discussed in more detail in section III.

III. Proposed Mechanism of Action of Organic Nitrate Esters on a Cellular Level

Organic nitrate esters have been observed to exert biochemical actions that have been proposed to explain the pharmacological activity of these drugs. Today, the emerging consensus strongly favours the involvement of the intracellular second-messenger cGMP as the mediator of vascular smooth muscle relaxation elicited by organic nitrate esters. cGMP also seems to be an important mediator for the vascular smooth muscle-relaxing action of SNP, molsidomine, nitrite, and other related compounds. However, it is important to stress that these compounds are members of distinct groups of vasodilators that display some common features as well as differences regarding their mode of action.

A. Cyclic Guanosine 3',5'-Monophosphate System

Although it is beyond the scope of the present review to describe all of the details of the cGMP system, it seems appropriate to give a general overview of the basic components of this second-messenger system and to stress the points of particular interest regarding the mechanism of action of organic nitrate esters and tolerance induction. For a more thorough discussion of the many functions of the cGMP system in different biological systems, interested readers are referred to previous reviews of this topic (Waldman and Murad, 1987; Tremblay et al., 1988; Lincoln, 1989).

After Sutherland and and Rall (1960) discovered the intracellular second-messenger cAMP, another cyclic nucleotide, cGMP, was isolated from mammalian sources (Ashman et al., 1963). The general arrangement of the cGMP system shows many similarities to the cAMP system, although important discriminating features do exist. A general scheme summarising the important components of the cGMP system is shown in figure 3.

cGMP is formed from guanosine 5'-triphosphate by the enzyme guanylate cyclase [guanosine 5'-triphosphate pyrophosphate (cyclising), EC 4.6.1.2], of which there seems to be at least three different forms: (*a*) soluble guanylate cyclase, (*b*) membrane-bound guanylate cyclase activated by ANPs and various egg peptides, and (*c*) guanylate cyclase associated with the cytoskeleton and activated by *Escherichia coli* heat-stable enterotoxin and calcium (Mittal and Murad, 1977; Klumpp and Schultz, 1982; Schulz et al., 1989; Waldman et al., 1986a,b; Garbers, 1989). Of these enzymes, the soluble form is considered to be the major target for activation by organic nitrate esters. Soluble guanylate cyclase has

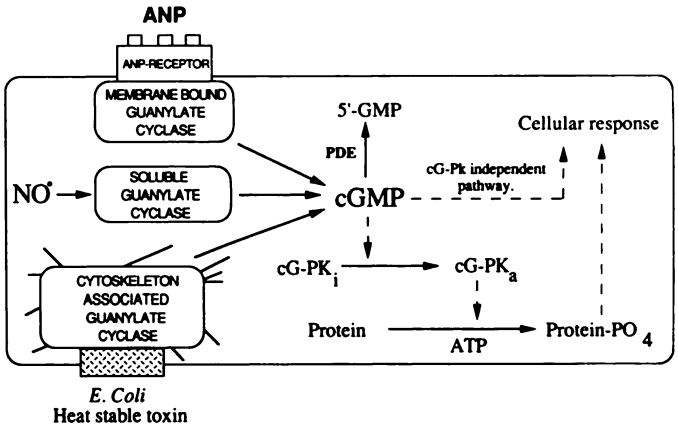

FIG. 3. Diagram of the basic components of the cGMP system, including three major forms of guanylate cyclase of which soluble guanylate cyclase is considered to be activated by organic nitrate esters through formation of nitric oxide. PDE, phosphodiesterase; i, inactive enzyme; a, active enzyme.

been purified to apparent homogeneity from different sources, including bovine lung, rat lung, human platelets, and rat brain (Chhajlani et al., 1989; Wolin et al., 1982; Kamisaki et al., 1986a; Zwiller et al., 1981a). The enzyme exists as a dimer, and different molecular weights for the two subunits have been reported in the literature (Waldman and Murad, 1987). However, in rat lung, human platelets, and bovine lung, the enzyme has been characterised as a heterodimer with apparent molecular weights of the two subunits corresponding to 82 to 83 and 70 to 71 kD, respectively (Chhajlani et al., 1989; Kamisaki et al., 1986a; Koesling et al., 1988). Expression of enzymatic activity and stimulation by nitric oxide appears to require both subunits (Nakane et al., 1990; Buechler et al., 1991). The primary sequence of the two subunits of the rat and bovine lung enzyme has recently been reported (Koesling et al., 1988, 1990; Nakane et al., 1988, 1990). Furthermore, recent evidence obtained from cloning experiments suggests that there heterogeneity may exist among the heterodimeric forms of soluble guanylate cyclase, at least as far as rat and humans are concerned (Yuen et al., 1990; Chhajlani et al., 1991).

Purified guanylate cyclase has been shown to contain heme (Gerzer et al., 1981, 1982), which is important for the activation of the enzyme by nitric oxide and nitric oxide-releasing agents, including organic nitrate esters. The heme moiety may be lost during purification of the enzyme, resulting in a heme-deficient molecule that is insensitive to activation by nitric oxide; reconstitution of the enzyme with heme restores the capacity for activation by these compounds (Craven and DeRubertis, 1978a, 1983; Ignarro et al., 1982, 1986a; Ohlstein et al., 1982). Furthermore, it also has been shown that guanylate cyclase can be activated by protoporphyrin IX, the demethylated precursor of heme (Ignarro et al., 1982; Ohlstein et al., 1982; Wolin et al., 1982). Electron paramagnetic resonance studies have demonstrated that the binding of nitric oxide to heme results in a structural

change of the heme with a displacement of the iron atom from the plane of the protoporphyrin molecule (Kon and Kataoka, 1969; Morse and Chan, 1980). Based on these findings it was suggested that activation of guanylate cyclase by nitric oxide is due to a conformational change of the heme prosthetic group, leading to a structure with the Fe^{2+} atom protruding out of the plane of the protoporphyrin molecule (Wolin et al., 1982; Ignarro et al., 1984b). This nitric oxide-heme complex shows a resemblance to protoporphyrin IX, and, therefore, the activation of guanylate cyclase by nitric oxide and protoporphyrin IX may share a common pathway.

Thiol compounds and thiol-modifying agents exert pronounced effects on guanylate cyclase activity, which is of particular significance when considering organic nitrate esters. Without going into all of the experimental evidence for the different possible alternatives, it seems that three important interactions between thiols and guanylate cyclase may exist. First, thiol groups seem to be located on the enzyme, and these are important for the regulation of enzyme activity, probably through reversible formation of disulfide bridges between juxtaposed SH groups (Craven and DeRubertis, 1978b; Ignarro et al., 1981a; Braughler, 1982, 1983; Brandwein et al., 1981; Kamisaki et al., 1986b). Second, thiol groups may facilitate the generation of reactive intermediates from organic nitrate esters and related compounds (Heppel and Hilmoe, 1950; Ignarro et al., 1980, 1981b; Ignarro and Gruetter, 1980; Gruetter et al., 1981b). The reactive intermediates formed may be either nitric oxide or S-nitrosothiols; these can function as the ultimate activators of guanylate cyclase, probably via an interaction with the heme moiety of the enzyme as described above. Third, thiols may also directly facilitate the formation of nitrosyl-heme complexes by acting as reducing agents and thus maintaining the heme iron in the reduced (Fe^{2+}) state (Craven and DeRubertis, 1978a, 1983; Kon, 1968).

Particulate guanylate cyclase, like soluble guanylate cyclase, appears to be widely distributed among different phyla and in different tissues. A particularly rich source of the particulate enzyme is sea urchin spermatozoa, and this material has been used for purification of the enzyme to apparent homogeneity (Garbers, 1976; Radany et al., 1983). Particulate guanylate cyclase has also been purified from rat lung (Waldman et al., 1983). Furthermore, it has been shown that the ANP receptor copurifies with guanylate cyclase, indicating that the ANP receptor and particulate guanylate cyclase are contained in a single transmembrane protein (Kuno et al., 1986). The ANP receptors coupled to particulate guanylate cyclase apparently belong to the ANP_A and ANP_B subtypes. Recently, the particulate guanylate cyclase-receptor complex has been cloned from sea urchin spermatozoa and rat brain, and the amino acid sequence has been deduced (Singh et al., 1988; Chinkers et al., 1989). The intracellular domain of the protein was found to possess one region homologous to protein kinases and one homologous to soluble guanylate cyclase. The protein kinase domain is essential for transmembrane signaling, probably by functioning as a regulatory element (Chinkers and Garbers, 1989).

It is generally believed that the cGMP formed from either the soluble or the particulate guanylate cyclase exerts its main action through activation of cG-Pk, at least as far as vascular smooth muscle and platelets are concerned. cG-Pk belongs to the serine/threonine protein kinases and exists as several isoenzymes with different chemical characteristics. Presently, two major classes of cG-Pk have been characterised, and these two forms have been designated type I and type II. The type I form has been further subdivided into type Ia and type Ib. Type Ia has been purified to apparent homogeneity and is well characterised (see reviews by Kuo et al., 1978; Lincoln and Corbin, 1983). This isoform of the enzyme is a dimer composed of two identical subunits, each containing a regulatory domain and a catalytic domain (Kuo, 1980; Edelman et al., 1987). Binding of cGMP to the cG-PK results in activation of the enzyme, but unlike the cAMP-dependent protein kinase, the subunits do not dissociate. Recently, a novel isoform of cG-Pk was isolated and characterised from bovine aorta (Lincoln et al., 1988b; Wolfe et al., 1989a). This enzyme, designated type Ib, exhibits similar, although clearly distinct, characteristics compared with type Ia. Because the type Ib is abundant in tissues rich in smooth muscle, it is possible that this isoform of the enzyme plays an important role in mediating the effect of cGMP in smooth muscle tissue, e.g., after stimulation with organic nitrate esters and ANP. The type Ib cG-Pk seems to undergo endogenous proteolysis to yield a monomeric form which is catalytically active (Wolfe et al., 1989b). The type II form of cG-Pk has been isolated from intestinal brush border and appears to exist as a monomer and exhibits several physical and chemical properties that are distinct from those of the type I enzyme (deJonge, 1981). Several different proteins that are phosphorylated by cG-Pk have been described, although the functional significance of these different proteins in regulating cell functions remains unclear.

The action of cGMP is terminated by opening of the 3′,5′-cyclic phosphoester bond yielding 5′-cGMP which is essentially inactive as far as activation of cG-Pk is concerned. This inactivation of cGMP is catalysed by phosphodiesterases. The cyclic nucleotide phosphodiesterases form a large family of isoenzymes differing in substrate specificity, allosteric regulation, and tissue distribution (Beavo and Reifsnyder, 1990). Five distinct families of phosphodiesterases have been identified, and one of these constitutes the cGMP-specific family (Beavo and Reifsnyder, 1990). Furthermore, selective inhibitors of some of these different cyclic nucleotide phosphodiesterase families have been synthesised and, two different inhibitors of the cGMP-specific family have been

identified, namely, dipyridamole and zaprinast (Weishaar et al., 1986; Gillespie and Beavo, 1989). These compounds have proved to be valuable tools in elucidating the mechanisms of action of organic nitrate esters.

1. Physiological regulators of the cyclic guanosine 3′,5′-monophosphate system. Delineation of the cGMP system as a probable mediator of the cardiovascular effects of organic nitrate esters sparked an intense interest in finding possible physiological regulators of the cGMP system with effects similar to organic nitrate esters. In 1980, Furchgott and Zawadzki reported that relaxation of vascular smooth muscle by acetylcholine required the presence of an intact endothelial cell layer and was due to release of a diffusible factor from the endothelium. It was shown that various other agents, including ATP, ADP, bradykinin, and calcium ionophore, could induce similar endothelium-dependent responses, and the relaxing substance released from the endothelium was named EDRF (Cherry et al., 1982). Independent reports from several laboratories showed that the EDRF-induced relaxation was associated with an increase in cGMP (Holzmann, 1982; Rapoport and Murad, 1983; Diamond and Chu, 1983; Furchgott et al., 1984; Ignarro et al., 1984a), and it was also found that EDRF could activate purified guanylate cyclase (Ignarro et al., 1986b; Mülsch et al., 1988a). In addition to inducing vascular smooth muscle relaxation, EDRF was also found to inhibit platelet aggregation (Azuma et al., 1986; Furlong et al., 1987; Alheid et al., 1987). The similarity of the activity profiles of EDRF, nitric oxide, and pharmacological agents that were thought to act via release of nitric oxide, possibly through the formation of some intermediate nitroso compound, led to the suggestion in 1986 that EDRF is nitric oxide or a nitroso compound (Ignarro et al., 1988; Furchgott, 1988). Chemical identification has also suggested that nitric oxide or possibly some nitroso compound is in fact EDRF (Palmer et al., 1987; Ignarro et al., 1987; Myers et al., 1990). Several extensive reviews of the topic of endothelium-dependent relaxation have been published (Furchgott and Vanhoutte, 1989; Angus and Cocks, 1989; Ignarro, 1989).

Recently, the concept of nitric oxide as an endogenous modulator of cell function was developed. This concept encompasses several cell types, including neurons, vascular smooth muscle cells, and white blood cells (Wood et al., 1990; Garthwaite et al., 1988; Bredt and Snyder, 1989; Bredt et al., 1990; Salvemini et al., 1990; Hibbs et al., 1988), which have all been shown to produce nitric oxide, probably from L-arginine- or L-arginine-containing precursors (Palmer et al., 1988; Sakuma et al., 1988; Schmidt et al., 1988). Of particular interest in the context of smooth muscle regulation are the recent findings that nerve-mediated relaxations induced in various smooth muscle tissues by electrical field stimulation appear to occur via activation of soluble guanylate cyclase by nitric oxide liberated from the tissue (Bult et al., 1990; Hobbs and Gibson, 1990; Tucker et al., 1990; Toda and Okamura, 1990; Ignarro et al., 1990; Axelsson et al., 1989a; Ahlner et al., 1991). The source of the nitric oxide, i.e., whether it is produced within neurons, smooth muscle cells, or other cell types in the preparations, is as yet unknown.

Another important physiological regulator of cGMP production and vascular smooth muscle function is ANP (Winquist et al., 1984; Rapoport et al., 1985; Ohlstein and Berkowitz, 1985). There are, however, important differences between the action of ANP and organic nitrate esters, because, as mentioned before, they stimulate cGMP production through activation of different forms of guanylate cyclase. This is, for instance, reflected in different sensitivities to inhibitors of cGMP production. Methylene blue and 6-anilino-5,8-quinolinedione (LY83583), which are potent inhibitors of soluble guanylate cyclase, have little or no effect on particulate guanylate cyclase and its activation by ANP, in either smooth muscle or ventricular heart muscle (Ohlstein and Berkowitz, 1985; Malta et al., 1988; Ekstam-Ljusegren, unpublished observations). The physiological role of ANP is not restricted to regulation of vascular smooth muscle function but encompasses effects on several other types of smooth muscle, kidney function, central nervous system activity, and the endocrine system (Leitman and Murad, 1987).

2. Cyclic guanosine 3′,5′-monophosphate system and its relation to the mode of action of organic nitrate esters.

a. VASCULAR SMOOTH MUSCLE. Today, there seems to be general scientific agreement regarding the important role played by the cGMP system in mediating the vascular effects of organic nitrate esters and also in mediating potentially important clinical effects on other target tissues such as platelets. It, therefore, seems appropriate to review in some detail the evidence on which this theory is based.

Serious efforts were started about 20 years ago to clarify the role of cGMP as a mediator of various cellular functions. It was gradually recognised that cGMP played a role in the regulation of smooth muscle tension, as had previously been shown for cAMP. However, it was first considered that cGMP was a promoter of smooth muscle contraction. This assumption was based on the observation that various smooth muscle contractants, including acetylcholine, serotonin, histamine, K^+, bradykinin, and prostaglandin $F_{2\alpha}$, were able to cause moderate increases in the cGMP levels (Lee et al., 1972; Dunham et al., 1974; Clayman et al., 1975; Kadowitz et al., 1975). In contrast, other authors found a poor correlation between the cGMP increase and the contractile response in smooth muscle (Diamond and Hartle, 1974; Diamond and Holmes, 1975; Diamond and Hartle, 1976). It was subsequently reported that GTN, SNP, sodium nitrite, and other smooth muscle relaxant agents also increased the tissue level of cGMP, which suggested that cGMP

plays a role in smooth muscle relaxation (Katsuki et al., 1977, Schultz et al., 1977). In appreciation of the view that the vasodilation induced by organic nitrate esters is the prime mechanism explaining their beneficial effect in the treatment of ischemic heart disease, it was a natural step to investigate the role of cGMP as a mediator of these effects.

To prove a causal relationship between an intracellular signal pathway and a response elicited by a defined stimulus in a particular tissue, one route could be to fulfill the classical criteria as originally set up by Sutherland and coworkers (1968) for cAMP. The first of these criteria states that a quantitative relationship should exist between the concentration of drug (or hormone) applied to the tissue, the tissue concentration of signal molecule, and the response. The second criterion is that changes in the concentration of the signal molecule should precede the response. Third, agents inhibiting the inactivation of the signal molecule should potentiate the effect of the stimuli, both concerning response and concentration of signal molecule. According to the fourth criterion, exogenous application of the signal molecule to a tissue specimen should mimic the response in the tissue elicited by the defined stimuli. Needless to say, permeability of the signal molecule across the plasma membrane must be carefully considered and, often, use of membrane-penetrating derivatives and/or permeabilisation of the tissue may be required, which may complicate the interpretation of the results. The fifth criterion indicates that enzymes involved in the formation of signal molecules should respond to the same stimuli, both in intact tissue and in broken cell preparations; if significant exceptions do occur, they must be properly explained. To these original criteria might be added that blocking of the generation of the signal molecule should also block the response of the tissue to the stimuli. Experimental studies performed with the above criteria in mind have proved helpful in delineating the role of the cGMP system in mediating the pharmacodynamic effects of organic nitrate esters. This is true, particularly, with regard to the vasodilatory action, which is considered to be the most important effect of this group of drugs in the treatment of angina pectoris and heart failure.

Several in vitro studies have shown a close correlation regarding the concentration-effect relationship between vascular smooth muscle relaxation and increase in cGMP. This relationship has been established for various blood vessels from different species and for different organic nitrate esters: bovine mesenteric artery for GTN, GDN, and glyceryl mononitrate (Axelsson et al., 1979, 1981); canine mesenteric artery for nicorandil and GTN (Endoh and Taira, 1983; Shibata et al., 1986); bovine coronary artery for GTN (Kukovetz et al., 1979), ISDN (Galvas and DiSalvo, 1983), and nicorandil (Holzmann, 1983; Schmidt et al., 1985); canine coronary artery, renal artery, and femoral artery for GTN (Shibata et al., 1986); rat aorta for GTN and IS-5-MN (Keith et al., 1982; Müller-Beckmann et al., 1984); rabbit aorta and rabbit vena cava for IS-5-MN (Matsuoka et al., 1985); bovine mesenteric vein for GTN (Axelsson, 1984); and human vena saphena magna for GTN (Ahlner et al., 1986a,b). Thus, there seems to be little doubt that the vascular smooth muscle relaxation induced by organic nitrate esters is accompanied by an increase in tissue cGMP level, regardless of the type of drug used and regardless of vascular type. However, concerning the absolute relationship between the degree of vascular relaxation evoked and the increase in cGMP level, different studies vary greatly, even when the same vessel and the same organic nitrate ester are used. This is also obvious when comparing the EC_{50} values obtained by different authors for a particular nitrate ester in similar types of vessels. One factor that seems to be of crucial importance is the type of contractile agent used to induce tension. Another factor that may also substantially influence the experimental results is the type of material(s) used in the manufacture of the experimental equipment; some materials may adsorb/absorb and retain the drug to a considerable extent (see preceding section). Furthermore, the age of the individual from which the vessel specimens were obtained may be of importance, and there may also be regional differences in one and the same vascular bed, explaining the apparent discrepancies.

Organic nitrate esters induce prompt relaxation in isolated vascular smooth muscle, and near maximal relaxation, which is attainable with a certain concentration of the drug, usually occurs within 1 min. It has been shown in various types of vascular tissues (veins, peripheral arteries, and coronary arteries) and for different organic nitrate esters, that this relaxation is preceded by an increase in tissue cGMP level, although the time-effect relationship varies between different vascular tissues (Axelsson et al., 1979; Kukovetz et al., 1979; Gruetter et al., 1981a; Keith et al., 1982; Holzmann, 1983; Rinaldo and Cingolani, 1983; Müller-Beckmann et al., 1984; Edwards et al., 1984; Matsuoka et al., 1985; Shibata et al., 1986). Proving a similar temporal relationship between relaxation and cGMP increase under in vivo conditions is, of course, much more difficult. However, in one study in which microwave fixation of rat coronary artery was used the cGMP increase was found to precede the increase in coronary blood flow (Kobayashi et al., 1980).

Results of in vitro studies show that the shape of the time curve for cGMP increase is dependent on the concentration of the organic nitrate ester used. Drug concentrations in the micromolar range elicit a sharp peak in tissue cGMP level within 1 to 2 min. Thereafter, the cGMP level gradually declines and reaches a lower steady-state level, which is maintained for a considerable time (Kukovetz et al., 1979; Axelsson et al., 1979; Axels-

son, 1984; Müller-Beckmann et al., 1984; Matsuoka et al., 1985). This peak in cGMP is abolished when lower concentrations of drug are used (Kukovetz et al., 1979; Edwards et al., 1984). As might be expected, the peak in cGMP level seems to be related to a higher rate of relaxation and may also affect the maximal relaxation obtained (Kukovetz et al., 1979; Matsuoka et al., 1985).

The potentiation of vascular smooth muscle relaxation and cyclic nucleotide levels by various inhibitors of cyclic nucleotide phosphodiesterases has been reported for different stimuli. Both zaprinast and dipyridamole, which are selective inhibitors of cGMP-specific phosphodiesterase, have been shown to potentiate the relaxation elicited by organic nitrate esters in various isolated vascular smooth muscles (Kukovetz et al., 1979; Holzmann, 1982; Shibata et al., 1986; Ahlner et al., 1985, 1986a).

In addition to the established concentration-effect and time-effect correlation for relaxation and cGMP increase established for organic nitrate esters, several authors have found that plasma membrane-penetrating cGMP analogues (chiefly 8-bromo-cGMP) are able to induce relaxation in vascular smooth muscle preparations and that this relaxation is potentiated by phosphodiesterase inhibitors (Schultz et al., 1979; Kukovetz et al., 1979; Napoli et al., 1980; Axelsson, 1984; Ahlner et al., 1986a; Saeed et al., 1987). It has been shown that 8-bromo-cGMP is a potent activator of cG-Pk, even more potent than native cGMP, which probably explains its vascular smooth muscle-relaxing properties (Kuo et al., 1974; Axelsson et al., 1980; Lincoln, 1983). However, cyclic nucleotide analogues may also exert some phosphodiesterase-inhibiting action. This may cause elevation of endogenous cyclic nucleotide levels (of both cAMP and cGMP) which could complicate interpretation regarding the exact mechanisms of action of these derivatives (Wells et al., 1975; Axelsson, 1984).

Activation of guanylate cyclase by organic nitrate esters in broken cell preparations is a more complicated matter. Significant guanylate cyclase activation in crude tissue homogenate or crude soluble fraction can only be obtained with high concentrations of organic nitrate esters (micromolar to millimolar range) and in the presence of exogenously added thiol compounds (Ignarro and Gruetter, 1980; Axelsson and Karlsson, 1984; Romanin and Kukovetz, 1988). It is not known why guanylate cyclase in crude broken cell preparations exhibits marked insensitivity to activation by organic nitrate esters compared with the situation in intact cells, in which guanylate cyclase activation occurs at very low concentrations of organic nitrate esters. Perhaps some kind of critical structural requirement exists regarding the arrangement of enzymes involved in metabolism of organic nitrate esters to nitric oxide and guanylate cyclase. Another possibility could be that endogenous inhibitors of guanylate cyclase and/or nitric oxide production are liberated from intracellular compartments during the preparation of cellular homogenates.

With regard to the concentration-effect relationship as estimated by in vitro studies of isolated vascular specimens, there seem to be important differences between GTN and other organic nitrate esters.

The mechanisms behind the two different components of the concentration-effect curve for GTN are not clear, although both seem to be mediated through an elevation in cGMP (Ahlner et al., 1987a; Malta, 1989). Inhibitors of cGMP generation, such as methylene blue or 6-anilino-5,8-quinolinedione (LY83583), inhibit both components (Axelsson et al., 1989a,b; Malta, 1989). However, the inhibition of the "high affinity" component is noncompetitive with regard to GTN, whereas the inhibition of the "low affinity" component is competitive. This indicates that these inhibitors have different mechanisms of action in relationship to the low and high affinity components (Malta, 1989). Because the high affinity component probably more closely represents the relevant drug concentration obtained in vivo, the question of how tolerance affects the different components is also of obvious importance. Finding an answer to this question may also help to clarify possible differences in the mechanisms underlying the different components.

"High dose" tolerance (50 μM GTN, 1 h) to GTN totally abolished the high affinity component of the concentration-effect curve in arteries, whereas in veins the concentration-effect curve was shifted to the right, and the contribution of the low affinity component was substantially reduced in favour of the high affinity component (Rösen et al., 1987). We have shown that induction of low dose tolerance in bovine mesenteric arteries (0.1 nM GTN, 2 h) preferentially reduced the high affinity component and had only a minor effect on the low affinity component, whereas high dose tolerance (100 μM GTN, 2 h) substantially reduced both the low and high affinity components (Ahlner et al., 1987b). However, in our study the biphasic appearance of the concentration-effect curve was discernible even after high dose tolerance. We also have found that tolerance to butanol-1,2,3,4-tetranitrate selectively abolishes the high affinity component of the concentration-effect curve for GTN (Axelsson et al., in preparation). These findings support the assumption that the different components are mediated, at least partly, through separate mechanisms.

In addition to the aforementioned characteristics of the biphasic concentration-effect relationship for GTN, there are differences between the high affinity and the low affinity components with regard to sensitivity to pertussis toxin. Pertussis toxin (from *Bordella pertussis*) is a widely used pharmacological tool, used to establish the involvement of certain types of guanine regulatory proteins in cellular signal transduction pathways (Milligan, 1988; Casey and Gilman, 1988; Ui and Katada, 1990; Reisine, 1990). ADP ribosylation of susceptible G-pro-

teins by pertussis toxin induces characteristic changes in their function, resulting in a disturbed signal transduction. We have shown that incubation of bovine mesenteric arteries in vitro with pertussis toxin significantly reduced the relaxant effect of GTN (Ahlner et al., 1988a). The high affinity component was markedly more sensitive to inhibition by pertussis toxin than was the low affinity component. Furthermore, the effect of pertussis toxin on relaxation was accompanied by an abolished cGMP response to GTN in the concentration range representing the high affinity component, and the effect of pertussis toxin was antagonised by a polyclonal antibody directed against pertussis toxin (Ahlner et al., 1988a). The effect of pertussis toxin appears to be specific for GTN, because relaxations induced by sodium nitrite, ISDN, IS-5-MN, and SNP were not affected by pretreatment of the arteries with pertussis toxin (Axelsson et al., 1989b; Torfgård et al., 1990a).

The above discussion suggests that vascular smooth muscle relaxation induced by GTN exhibits certain unique characteristics, i.e., biphasic concentration-effect relationship as compared to other organic nitrate esters and related vasodilators. Although cGMP seems to be important as a mediator of both components, one can only speculate as to the functional basis for these two components. It might be speculated that the characteristic biphasic concentration-effect curve for GTN is due to activation of different pools of soluble guanylate cyclase (Ahlner et al., 1987b). The activation of these different pools may, at least in part, proceed through separate and distinct pathways and with a pertussis toxin-sensitive regulatory protein involved in activation of the pathway representing the high affinity component. However, the effect of pertussis toxin may not be related to modification of a regulatory protein in the traditional manner. It has been shown that the site of ADP ribosylation of G-proteins by pertussis toxin is a conserved cysteine residue located four amino acids from the COOH terminus of the α-subunit of the proteins (Milligan, 1988), and thiol compounds may also function as ADP ribosylation substrates for pertussis toxin in vitro (Lobban and van Heyningen, 1988). Speculatively, the effect of pertussis toxin on the relaxation and cGMP production may be due to inactivation of critical thiol groups necessary for the activation of soluble guanylate cyclase by GTN. These thiol groups could be located either on the guanylate cyclase itself or on proteins/peptides involved in the production of guanylate cyclase-activating intermediates (e.g., S-nitrosothiols) from GTN. The apparent greater sensitivity of the high affinity component to tolerance induction may also point to a preferential involvement of thiols in this signal pathway. The possibility also exists that activation of soluble guanylate cyclase by GTN proceeds through several different thiol-dependent pathways, each dependent on the presence of a specific thiol compound. The role played by thiol compounds in the activation of the cGMP system by organic nitrate esters and in tolerance induction to organic nitrate esters will be discussed more thoroughly later.

Another possible explanation for the biphasic concentration-effect curve produced by GTN is that different isoenzymes of guanylate cyclase may be activated. In view of the recently reported heterogeneity among soluble guanylate cyclase in rat (Yuen et al., 1990) and man (Axelsson et al., in preparation), the possibility might be considered that the different forms may be differentially regulated. Furthermore, nitric oxide activation of washed and partially purified particulate forms of guanylate cyclase has been demonstrated (Lad and White, 1979; Waldman et al., 1982; Waldman and Murad, 1987). The interpretation of these results may, however, be complicated because of possible contamination of the particulate guanylate cyclase preparations by the soluble isoenzyme. It is also possible that the particulate form of guanylate cyclase associated with the cytoskeleton may be activated by organic nitrate esters and, thus, contribute to the biphasic response. This form of guanylate cyclase is preferentially found in intestinal mucosa and is activated by *E. coli* heat-stable enterotoxin (Waldman et al., 1986a). Little is known about the occurrence or function of this enzyme in tissues other than intestinal mucosa or about its possible activation by agents other than *E. coli* heat-stable toxin. Interestingly, the signal transduction between the receptor for *E. coli* heat-stable enterotoxin and the effects mediated by this cytoskeleton-associated guanylate cyclase appears to be sensitive to pertussis toxin; this is not the case for the ANP-mediated effects in vascular smooth muscle (Epstein et al., 1986; Ekstam Ljusegren et al., 1990).

The speculation that the different components of the concentration-effect curve for GTN may be explained by activation of different pools or isoenzymes of guanylate cyclase may also imply that a cellular compartmentalisation exists regarding the cGMP formed by these different mechanisms. This may, in turn, lead to activation of distinct cellular processes (e.g., reduction in cytosolic free calcium concentration, phosphorylation of contractile proteins, hyperpolarisation), ultimately leading to vascular smooth muscle relaxation.

Of some interest in relation to the biphasic concentration-effect relationship for GTN is the report of a similar biphasic relationship for endothelium-dependent relaxation of vascular smooth muscle by acetylcholine (Rubanyi and Vanhoutte, 1987). From this, and also from other findings, it was inferred that there may be two different endothelium-derived factors (i.e., EDRF and endothelium-derived hyperpolarising factor) that induce vascular smooth muscle relaxation through different mechanisms (Chen et al., 1988; Feletou and Vanhoutte, 1988). Recent evidence suggests that both of these may be identical with nitric oxide, which would thus act

through two different mechanisms to induce vascular smooth muscle relaxation (Tare et al., 1990).

b. PLATELETS. It seems clear that organic nitrate esters inhibit platelet function. This has been shown in vitro for several different organic nitrate esters, including GTN, ISDN, and isosorbide mononitrate (Schaffer et al., 1980; Loscalzo, 1985; Ahlner et al., 1985; Gerzer et al., 1988; Negrescu et al., 1990). The inhibition of aggregation is correlated with an increase in cGMP, and both the antiaggregatory action and elevation in cGMP are potentiated by dipyridamole (cGMP-phosphodiesterase inhibitor) and N-acetylcysteine (Loscalzo, 1985; Ahlner et al., 1985; Stamler et al., 1988). In addition, it has been suggested that other aspects of platelet function, such as adhesion and the release reaction, may be regulated by cGMP (Takai et al., 1981; Yamanishi et al., 1983; Radomski et al., 1987; Sneddon and Vane, 1988). It also seems possible that organic nitrate esters exert similar effects on platelets, although this remains to be determined.

c. MYOCARDIUM. The beneficial effects of organic nitrate esters on the ischemic myocardium are thought to result from dilation of coronary arteries and peripheral vessels, leading to an improved nutritional and oxygen status in the myocardial tissue, together with a reduction in work load. These drugs may also have important direct effects on the myocardial cells. It has been clearly demonstrated that 8-Br-cGMP and SNP reduce the lactate content in spontaneously beating ischemic rat atrial tissue in vitro (Laustiola et al., 1983a,b). GTN in the presence of cysteine has been shown to have a similar effect, whereas GTN alone was ineffective in reducing lactate levels in ischemic rat atrium and was also without effect on the cGMP level (Laustiola et al., 1983c). In addition to a reduction in lactate levels, an increase in tissue energy charge and a reduction in nicotinamide adenine nucleotide (reduced form) levels have been demonstrated, indicating an overall improvement of metabolic parameters by cGMP during ischemia (Vuorinen et al., 1984; Laustiola et al., 1983b; Laustiola et al., 1984). Similar results have been obtained in Langendorff-perfused rat heart (Laustiola, 1985). We have recently found that SNP and ANP reduce lactate production in ischemic rat left ventricle in vitro (Ekstam Ljusegren et al., in preparation). This effect of SNP and ANP was mimicked by 8-Br-cGMP. In contrast, GTN, in the concentration range 1 nM to 1 mM, was found to increase lactate accumulation in rat ventricular muscle, both under normoxic conditions and during hypoxia (fig. 4; Ekstam Ljusegren et al., in preparation). The reduction in lactate accumulation induced by SNP and ANP in ischemic myocardium was correlated with an increase in cGMP, whereas GTN did not affect cGMP levels in myocardial tissue (Ekstam Ljusegren et al., unpublished observations). The effect of other organic nitrate esters, apart from GTN, on myocardial metabolism remains to be studied. It would also be of considerable interest to determine the effects of organic nitrate esters on myocardial metabolism under in vivo conditions. However, such studies are difficult to perform, because any direct effect on myocardial metabolism by these drugs may be masked by their concurrent effects on the coronary and peripheral vascular beds. This would lead to an improved myocardial metabolism through increased blood delivery to the ischemic myocardium, combined with a reduction in workload.

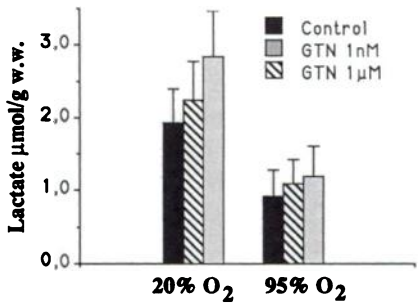

FIG. 4. Effect of 1 nM or 1 mM GTN on lactate production in isolated rat ventricular heart muscle. The tissue specimens were incubated in glass vials for 60 min in Krebs bicarbonate buffer solution (95% O_2 + 5% CO_2) and thereafter transferred to new vials containing Krebs bicarbonate solution equilibrated with either 95% O_2 + 5% CO_2 or 20% O_2 + 5% CO_2 + 75% N_2. The lactate content in the tissue was measured after 5 min. Means ± SEM; $n = 6$.

3. Organic nitrate esters, cyclic guanosine 3',5'-monophosphate, and the effect on cellular calcium homeostasis and contractile proteins. In view of the central role played by the intracellular free Ca^{2+} concentration in the regulation of smooth muscle tension, a logical step would be to examine the effect of organic nitrate esters on cellular calcium turnover. Such studies have been undertaken by several authors. Grün and coworkers (Grün and Fleckenstein, 1972; Weder and Grün, 1973) suggested that GTN exerted a calcium-antagonistic action in vascular smooth muscle and, therefore, showed mechanistic similarities to the calcium channel blockers. In subsequent studies in which radiolabeled calcium was used, SNP was reported to reduce calcium influx and/or increase calcium efflux in various vascular smooth muscle preparations, and these effects were suggested to be the major mechanisms of action of these drugs (Zostér et al., 1974; Kreye et al., 1975; Zostér et al., 1977; Hester et al., 1979). A reduction in calcium influx by GTN was also reported in rabbit pulmonary artery by Thorens and Haeusler (1979), although this effect was only seen with high (millimolar) concentrations of GTN. In intact bovine mesenteric artery in vitro, 1 µM GTN was found to decrease phenylephrine-stimulated calcium influx (Ahlner et al., 1990). However, at a lower concentration (0.1 nM) GTN exerted a substantial relaxant effect on bovine mesenteric artery but had no effect on calcium influx (Ahlner et al., 1990).

In opposition to these studies, GTN and SNP, even at high concentrations, were not found to exert any effect

on transmembrane calcium fluxes in canine coronary artery and human umbilical vessels (Nakazawa and Imai, 1981; Ozaki et al., 1981). Therefore, organic nitrate esters seem to exert some effects on calcium fluxes across the plasma membrane, at least in certain types of vascular smooth muscle. However, the functional significance of this effect is not clear, especially at lower and clinically relevant drug concentrations (i.e., nanomolar to micromolar range). One possible explanation for the varying results of the investigations discussed above may be that the methods available at present to study transmembrane calcium fluxes are not sensitive enough to allow reliable determination of small, but functionally important, changes in calcium fluxes.

Increased binding of calcium to intracellular stores in arterial smooth muscle has also been suggested to be of importance for the relaxant activity of organic vasodilators, e.g., SNP and cGMP, in various vascular smooth muscle preparations (Kreye et al., 1975; Lincoln, 1983; Twort and van Breemen, 1988; Ahlner et al., 1990). We have recently shown that IP_3-stimulated calcium release from microsomes is significantly reduced by GTN, both at low (0.1 nM) and high (1 mM) concentrations (Ahlner et al., 1990). This effect is transient and is mimicked by exogenously applied cGMP. Furthermore, cGMP and agents that stimulate cGMP synthesis, including GTN, have been shown to reduce phosphatidylinositol turnover in isolated vascular smooth muscle and platelets (Takai et al., 1981; Nakashima et al., 1986; Fujii et al., 1986; Rapoport, 1986; Ahlner et al., 1988b). This could suggest that a reduction in IP_3 production in, and consequently of calcium release from, intracellular stores may be an important mechanism by which cGMP and organic nitrate esters induce smooth muscle relaxation.

In a previous study in our laboratory, GTN was found to inhibit protein kinase C activation by contractile stimuli (Ahlner et al., 1988b). This effect could be due to several factors, including a decreased intracellular free calcium concentration and/or decreased formation of diacylglycerol, both of which are important activators of protein kinase C. Diacylglycerol and IP_3 are formed from membrane lipids through the action of phospholipase C, and it has been proposed that cGMP inhibits phospholipase C, possibly through modification of a guanine regulatory protein (Nakashima et al., 1986; Hirata et al., 1990). Evidence suggests that protein kinase C participates in the regulation of smooth muscle tension and platelet function, probably through phosphorylation of various proteins (Nishizuka, 1984; Movesian et al., 1984; Chiu et al., 1987; Hagiwara et al., 1987). Inhibition of protein kinase C would, therefore, facilitate relaxation of vascular smooth muscle and inhibition of platelet aggregation.

Attempts have been made to measure the effect of organic nitrate esters, and other agents causing cGMP elevation, on the intracellular concentrations of free calcium. The free intracellular calcium concentration can be indirectly estimated by determining the activity of calcium-dependent enzymes such as phosphorylase a (Gross and Mayer, 1974). Several agents known to increase cGMP, including GTN, have been proven to reduce the phosphorylase a activity in precontracted vascular smooth muscle (Axelsson et al., 1985; Johnson and Lincoln, 1985; Lincoln et al., 1988a). More direct estimation of the free intracellular calcium concentration has been attempted using intracellular calcium probes such as quin2 and fura-2. It was demonstrated, using this method, that cGMP, ANP, and SNP can reduce the intracellular calcium concentration in both vascular smooth muscle cells and platelets (Rashatwar et al., 1987; Lincoln et al., 1988a; Kawahara et al., 1984, MacIntyre et al., 1985; Mülsch et al., 1989c; Lockette et al., 1989; Morgan and Newby, 1989). GTN and ISDN have also been shown to inhibit the receptor-induced increase in cytosolic calcium in human platelets (Negrescu et al., 1990).

Simultaneous measurements of force and intracellular calcium concentrations have been performed in KCl-depolarised canine coronary arteries after treatment with GTN (Yanagisawa et al., 1989). These measurements showed a clear dissociation of GTN-induced relaxation from a decrease in intracellular Ca^{2+} because relaxation occurred without any reduction in intracellular Ca^{2+}. Furthermore, the curve describing the relationship between intracellular free [Ca^{2+}] and force was shifted to the right, indicating an effect of GTN on the contractile proteins (presumably a decrease in myosin light chain phosphorylation) (Yanagisawa et al., 1989). In support of this, studies of phosphorylation of endogenous proteins have indicated that cGMP and cGMP-elevating agents, such as SNP and EDRF, reduce the phosphorylation of myosin light chain, both in vascular smooth muscle and platelets, in addition to increasing the phosphorylation of a number of proteins with unknown functions (Rapoport et al., 1982, 1983a,b; Drazsnin et al., 1983; Kawahara et al., 1984).

However, thus far, similar investigations of the effect on protein phosphorylation of different organic nitrate esters remain to be performed. Studies are also needed to determine the effects of different organic nitrate esters on the intracellular calcium level. Various types of contractile agents, not only KCl depolarisation, which is highly unphysiological and is known to be markedly resistant to relaxation by organic nitrate esters (Axelsson, 1984; Ahlner, 1987) should be used.

In addition to the above described mechanisms by which organic nitrate esters and cGMP may regulate the intracellular calcium concentration, a stimulatory effect on the sarcolemmal calcium-pumping ATPase has been shown (Popescu et al., 1985; Furukawa and Nakamura, 1987; Ahlner et al., 1990). It was suggested that this effect was mediated by direct cGMP-dependent phos-

phorylation of the ATPase (Furukawa and Nakamura, 1987). However, it appears that the phosphorylated protein and the calcium-pumping ATPase are distinct molecular entities (Baltensperger et al., 1988).

In conclusion, there seem to be multiple sites of action at which cGMP can mediate the cellular effects of organic nitrate esters; some of these are summarised in fig. 5.

B. Other Suggested Mechanisms of Action of Organic Nitrate Esters

Early theories regarding the mechanism of action of organic nitrate esters suggested that this group of drugs might interfere with energy production and energy utilisation in vascular smooth muscle cells, thereby inducing dilation of blood vessels. Krantz and coworkers (1951) reported an inhibition of arterial ATPase activity by GTN and amyl nitrite. It was also found that high concentrations of GTN and related compounds uncoupled oxidative phosphorylation in rat liver mitochondria, and the relative potencies of the different agents in this regard correlated with their effectiveness as vasodilators (Needleman and Hunter, 1966). These findings confirmed the observation of Hunter et al. (1953) that treatment with mannitol hexanitrate reduced the rate of oxidative phosphorylation in rat liver mitochondria. GTN was also reported to reduce ADP-stimulated respiration in rat liver mitochondria (Boime and Hunter, 1971). This effect was only seen when nicotinamide adenine nucleotide-linked respiratory substrates were used. It was, therefore, suggested that GTN had a specific effect on the nicotinamide adenine nucleotide (reduced form) dehydrogenase domain of the electron transport chain. However, because GTN and the other nitrate esters were used in concentrations in the millimolar range, the interpretation of these results is difficult. Maximal effect on isolated blood vessels in vitro is seen when organic nitrate esters are used in the submicromolar range; the relevant in vivo concentration range is probably much lower. Furthermore, SNP, which exerts a smooth muscle-relaxing action resembling that of GTN, had no effect on the ATP level in rat aorta (Kreye et al., 1975), and it was concluded that metabolic inhibition was not the mechanism responsible for the vascular smooth muscle relaxation induced by this drug. In rabbit heart mitochondria, incubated under conditions similar to those prevailing in the ischemic heart, GTN was found to improve oxidative phosphorylation (Szekeres et al., 1978); similar findings were later reported for dog heart mitochondria (Suzuki et al., 1981). Based on these results, a direct effect of GTN on myocardial mitochondria was suggested to be an important mechanism explaining the antianginal effects of this drug.

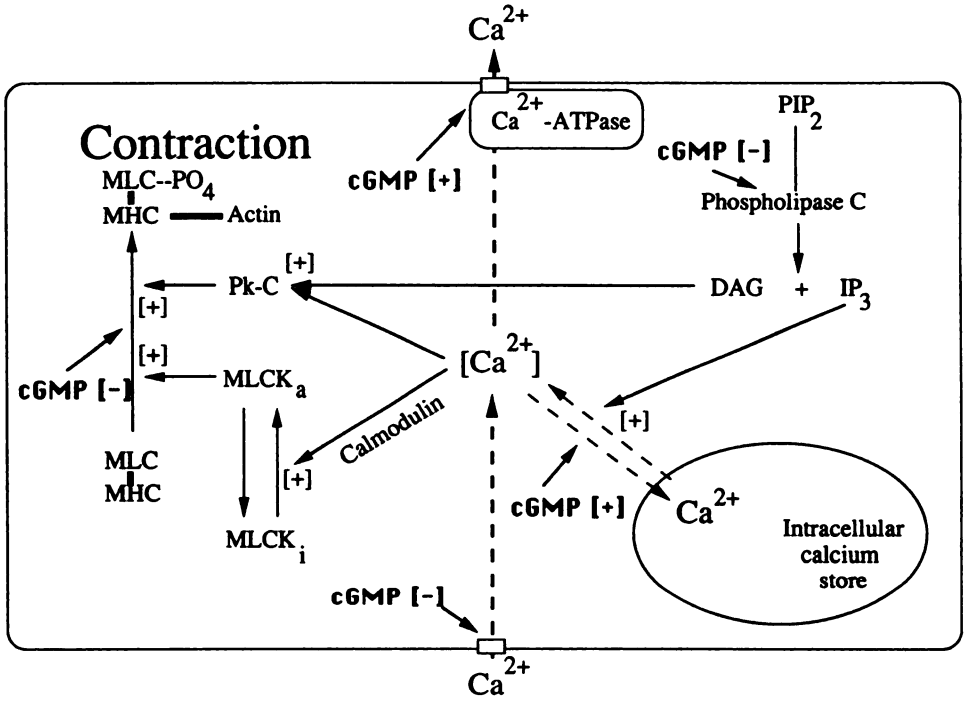

FIG. 5. Schematic drawing showing some of the cellular mechanisms suggested to be targets for cGMP and mediating vascular smooth muscle relaxation. cGMP stimulates [+] calcium efflux over the plasma membrane and uptake of calcium to intracellular calcium-storing compartments, while inhibiting [−] calcium influx over the plasma membrane and production of diacyl glycerol (DAG) and IP_3. The latter effect may result in decreased calcium liberation from intracellular stores and reduced myosin light chain (MLC) phosphorylation by protein kinase C (Pk-C) and calcium/calmodulin-activated myosin light chain kinase (MLCK). An inhibition of MLC phosphorylation by cGMP through other, as yet, unknown mechanisms is also indicated as a possibility. This would reduce actin-myosin interaction and, thus, reduce vascular smooth muscle contraction. Ca-ATPase, calcium-extrusion ATPase, MHC, myosin heavy chain; PIP_2, phosphatidyl inositol bisphosphate; i, inactive enzyme; a, active enzyme.

However, in these investigations, concentrations of GTN in the micromolar to millimolar range were used, making it difficult to evaluate the significance of the results under in vivo conditions. We have recently found that GTN reduces oxygen consumption in isolated rat ventricular muscle, and this effect was seen even when using GTN concentrations as low as 1 nM (fig. 6; Ekstam Ljusegren et al., in preparation). The mechanism behind this effect is unknown, although it seems unlikely that cGMP is involved, because GTN had no detectable effect on cGMP levels in isolated rat ventricular muscle.

1. The "nitrate receptor" theory. A very different concept concerning the mechanism of action of organic nitrate esters was put forward by Needleman and coworkers (Needleman et al., 1973; Needleman and Johnson, 1973). These authors found that chronic exposure of vascular smooth muscle to GTN induced a specific tolerance to the drug, somewhat in analogy to the well-known desensitisation of hormone receptors after prolonged exposure to their respective agonists. Based mainly on these findings, the existence of a specific nitrate receptor in the smooth muscle cell membrane was suggested. SH groups located on proteins constituting an integral part of the nitrate receptor, and on a so-called "common vasodilator intermediate site," were considered to be of critical importance for GTN to exert its smooth muscle-relaxing effect. The putative nitrate receptor was not further characterised, nor was its mode of action. In a recent study in which radiolabeled GTN was used, no evidence could be obtained for a specific GTN-binding site in bovine pulmonary vein (Kawamoto et al., 1988). Interaction with SH groups also appears to be the mechanism by which high concentrations of GTN and other organic nitrates exert their inhibitory action on certain SH-rich enzymes, such as monoamine oxidase and glyceraldehyde-3-dehydrogenase (Jakschik and Needleman, 1973).

SH groups appear to be of crucial importance in modulating the activity of soluble guanylate cyclase. This enzyme may function as a receptor for organic nitrate esters because it interacts with nitric oxide released from these drugs (see above).

2. Hyperpolarisation of the vascular smooth muscle cell.

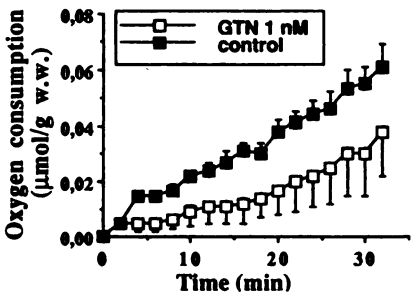

FIG. 6. Effect of GTN on oxygen consumption in isolated rat ventricular heart muscle incubated under normoxic conditions (Krebs bicarbonate buffer solution, 95% O_2 + 5% CO_2). Oxygen consumption was measured with Warburg technique. Means ± SEM; n = 3 to 6.

It is known that hyperpolarisation of isolated blood vessels causes relaxation (Kuriyama et al., 1982), and it has also been shown that the vasodilator effect of SNP is accompanied by membrane hyperpolarisation and a reduced membrane resistance in rabbit pulmonary artery and rabbit portal vein (Haeusler and Thorens, 1976; Ito et al., 1978). This has led to the assumption that the vascular smooth muscle relaxation induced by organic nitrate esters could, at least in part, be explained by hyperpolarisation. However, when GTN was examined for effects on membrane properties of smooth muscle from pig and canine coronary artery (Ito et al., 1980a,b), no such effects could be established. In contrast, GTN, as well as nitric oxide and EDRF, was recently reported to cause hyperpolarisation of guinea pig uterine artery (Tare et al., 1990). These findings may also suggest that the putative endothelium-derived hyperpolarising factor is identical with nitric oxide and that hyperpolarisation and cGMP elevation are both induced by nitric oxide.

3. K channel activation. Nicorandil is an organic nitrate ester with one nitrate ester group, and it has been shown that the vasodilation induced by nicorandil is accompanied by cGMP elevations (Endoh and Taira, 1983; Holzmann, 1983). It was also demonstrated that nicorandil was able to activate guanylate cyclase in broken cell preparations (Schmidt et al., 1985). From these results is was concluded that nicorandil had a similar mechanism of action as other organic nitrate esters, i.e., stimulation of vascular smooth muscle guanylate cyclase with subsequent cGMP production and muscle relaxation. Nicorandil also is a powerful potassium channel opener (Furukawa et al., 1981; Kajioka et al., 1990), and, therefore, it is apparent that nicorandil represents a hybrid molecule possessing both potassium channel-opening and guanylate cyclase-stimulating properties (Edwards and Weston, 1990). The relative importance of these actions of nicorandil for the relaxation of vascular smooth muscle is not presently clear.

4. Arachidonic acid metabolites and vascular actions of organic nitrate esters. Findings that have attracted considerable interest indicate that GTN stimulates prostacyclin synthesis in cultured endothelial cells (Levin et al., 1981) and in isolated human, rat, and bovine vascular preparations in vitro (Metha et al., 1983a,b; Wallis et al., 1982; Schrör et al., 1981, 1984; Rücker and Ahland, 1983; Tsai et al., 1989). It has also been found that thromboxane A_2 synthesis is inhibited by GTN (Schrör et al., 1981). Based on these observations, it was concluded that these actions are important mechanisms contributing to GTN-associated vasodilation, and they help explain its beneficial effect in the treatment of myocardial ischemia. Furthermore, it was also reported that inhibitors of prostaglandin synthesis blunt the relaxation induced by GTN in isolated perfused cat coronary artery (Ouyang and Reid, 1980) and the cardiovascular effects of GTN in dogs (Morcillo et al., 1980; Trimarco et al.,

1985) and in humans (Thadani and Kellerman, 1983). However, using in vivo animal models, other authors found no evidence that prostaglandins play a role in mediating the vascular actions of different organic nitrate esters, GTN, ISDN, etc. (Lippton et al., 1981; Feigen et al., 1978; Chapnik et al., 1977; Lippton et al., 1984; Nugent et al., 1982; Panzenbeck et al., 1984; Simonetti et al., 1985; Sakai et al., 1983; Winniford et al., 1984).

The conflicting results obtained in vivo may be explained, at least partly, by an effect of some of these inhibitors on the uptake or metabolism of GTN. However, investigations concerning the pharmacokinetic interaction between GTN and acetylsalicylic acid have shown an increased, not reduced, peak plasma concentration and an increased "AUC" for GTN after administration of acetylsalicylic acid (Rey et al., 1983; Weber et al., 1983). Similar findings were also found in isolated rabbit mesenteric and celiac arterial rings (Bennett et al., 1983). In a previous report from our laboratory, several inhibitors of the different branches (cyclooxygenase, lipoxygenase, and cytochrome P-450) of the arachidonic acid-metabolising pathway were tested with regard to their effect on GTN-induced relaxation in isolated bovine coronary and mesenteric arteries and bovine mesenteric vein (Bornfeldt and Axelsson, 1987). The effect of some of these inhibitors on cyclic nucleotide levels in the vascular tissue was also determined. The only statistically significant effect seen in this study was a potentiation of the GTN-induced relaxation after treatment with some of these inhibitors; the putative prostacyclin synthesis inhibitor tranylcypromine was most effective. None of these inhibitors had any detectable effect on cGMP or cAMP generation.

Based on the finding that indomethacin caused partial inhibition of the relaxation induced by low concentrations of GTN in arteries, Rösen et al. (1987) suggested that prostaglandins may be of importance in mediating the relaxation represented by the high affinity component discussed before. However, opposed to this finding, an involvement of prostaglandins in mediating either of the components could not be found in rat aortic rings (Malta, 1989).

Thus, considered together, these data do not support the idea that arachidonic acid metabolites play any major role in mediating the vascular effect of organic nitrate esters. However, it seems likely that some arachidonic acid metabolite(s) plays a modulatory role in the vascular effect of organic nitrate esters. On the other hand, different organic nitrate esters may have different effects on the production of vasoactive arachidonic metabolites, a matter which seems to have attracted limited interest so far.

IV. Modulation of Target Tissue Responses to Organic Nitrate Esters by Local Factors

A. Role of Tissue Biotransformation of Organic Nitrate Esters in Their Mechanism of Action

Several recent studies have yielded information consistent with the idea that organic nitrate esters act as prodrugs to release a pharmacologically active intermediate; SH-containing molecules, such as cysteine or glutathione, are apparently of vital importance for this reaction. This concept was introduced by Needleman and coworkers (Needleman and Krantz, 1964; Needleman et al., 1969) and subsequently elaborated upon by Ignarro and coworkers (1981b). The chemical identity of the intermediate formed is not entirely clear, although strong candidates are nitric oxide or some S-nitrosothiol compound. The formation of this intermediate seems to depend on biotransformation of the organic nitrate ester in or near the target tissue (e.g., vascular smooth muscle). We have shown that the rate of enzymatic denitration of various organic nitrate esters in homogenates of bovine mesenteric artery is correlated with their potency regarding cGMP elevation and smooth muscle relaxation (Wingren et al., 1981), thus indicating a connection between the action of organic nitrate esters and their denitration. Yeates and coworkers (1985) subsequently showed a good correlation between the reaction of cysteine and different organic nitrate esters and the vasodilator potency of these compounds. These authors also found a correlation between the rate of formation of nitrite ion and the pharmacological potency of the different organic nitrate esters. However, because nitrite and nitrate should only be considered as end products of the denitration process, and because rather high concentrations of nitrite and nitrate are required to induce vascular smooth muscle relaxation and to activate guanylate cyclase, direct measurements of the formation of intermediates (such as nitric oxide and S-nitrosothiols) from organic nitrate esters are required to substantiate the "prodrug hypothesis." Such measurements should also be combined with studies of the effect exerted by the organic nitrate ester. Another question that needs to be addressed is whether the formation of reactive intermediates from organic nitrate esters is enzymatic, nonenzymatic, or a combination of both.

Using an in vitro system, Feelisch and Noack (1987) showed nonenzymatic, temperature-dependent formation of nitric oxide from organic nitrate esters and SNP. The rate of nitric oxide formation correlated well with the potency of these compounds in activating guanylate cyclase. Furthermore, there seemed to be different nonenzymatic, SH-dependent pathways for the degradation of organic nitrate esters (Feelisch et al., 1988). This may, in some cases, lead to the generation of nitric oxide and subsequent guanylate cyclase activation, whereas in other cases nitrite is formed, apparently directly, without formation of nitric oxide as an intermediary product.

This latter pathway may thus be "futile" as far as guanylate cyclase stimulation is concerned.

Another approach to study the relationship between the metabolism of organic nitrate esters and their vasodilator effect has been to determine drug metabolites in vascular smooth muscle tissue during relaxation. Such studies have been performed for GTN, and these have shown a time-dependent increase in GDN formation which correlated with the decrease in tension (Brien et al., 1986). Subsequent investigations showed that the appearance of GDN metabolites was accompanied by an increase in tissue cGMP levels, and both the appearance of GDN and the increase in cGMP preceded the relaxation elicited by GTN (Brien et al., 1988; Kawamoto et al., 1990). A relationship between biotransformation of GTN and increase in cGMP levels has also been shown in cultured cells of both vascular and nonvascular origin (Bennett et al., 1989). Thus, good experimental evidence exists indicating biotransformation as a prerequisite for GTN-induced vascular smooth muscle relaxation. However, a similar temporal relationship between biotransformation and pharmacological effect for other organic nitrate esters remains to be established.

Denitration of GTN can give rise to two structurally different GDNs, i.e., 1,2-GDN and 1,3-GDN. It appears that biotransformation of GTN in vascular smooth muscle preferentially gives rise to 1,2-GDN, with the 1,2-GDN/1,3-GDN ratio ranging from 3 to 8, depending on the type of vascular smooth muscle (Kawamoto et al., 1987, 1990; Brien et al., 1988; Slack et al., 1989). In cultured cell lines it was found that the selective formation of 1,2-GDN is most pronounced at low concentrations of organic nitrate ester (<0.1 mM), whereas at higher concentrations this selectivity is lost (Bennett et al., 1988a, 1989). The concentration dependency regarding which isomer of GDN will be formed may possibly explain the results of Fung et al. (1984), who found that approximately equal amounts of 1,2-GDN and 1,3-GDN were in rat vascular smooth muscle of different origins, incubated in the presence of GTN.

It was recently shown that incubation of rabbit liver cytosol with GTN also resulted in the preferential formation of the 1,2-GDN metabolite, whereas in the microsomal fraction, the 1,3-GDN metabolite dominated (Lau and Benet, 1990). The ratio 1,2-GDN/1,3-GDN was clearly dependent on the GTN concentration, at least in the cytosolic fraction, and the formation of the two metabolites also showed different sensitivity to inhibitors of glutathione S-transferase. Based on these results it was concluded that the formation of 1,2-GDN and 1,3-GDN is catalysed by different isoenzymes of glutathione S-transferase (Lau and Benet, 1990). Because several different isoenzymes of glutathione S-transferase have been purified from human aorta (Tsuchida et al., 1990), and because inhibitors of this enzyme have been shown to antagonise GTN-induced vascular smooth muscle relaxation (Yeates et al., 1989), it is likely that glutathione S-transferase is also involved in the degradation of GTN to GDN in vascular smooth muscle. However, other enzymes are probably also involved in target tissue biotransformation of organic nitrate esters.

It was recently demonstrated in rat liver that nitric oxide is formed from GTN via a cytochrome P-450-dependent pathway (Servent et al., 1989; McDonald and Bennett, 1990), suggesting that cytochrome P-450 could be of importance for the pharmacological effects of organic nitrate esters. However, in a previous study we were unable to demonstrate any effect of the cytochrome P-450 inhibitor SKF-525A on GTN-induced relaxations in various types of isolated vascular smooth muscles (Bornfeldt and Axelsson, 1987); the cytochrome P-450 inhibitor metyrapone was also found to be completely without effect on GTN-induced relaxation in bovine mesenteric artery (Torfgård et al.,in preparation). This could indicate that cytochrome P-450 is not of major importance in vascular smooth muscle relaxation elicited by GTN. However, results obtained with inhibitors of cytochrome P-450 must be interpreted with caution, because it is known that there exist several different isoenzymes of cytochrome P-450, exhibiting vastly different sensitivity toward compounds classically considered to be cytochrome P-450 inhibitors (Murray and Reidy, 1990). It would be of interest to investigate the effect of drug-induced induction (e.g., phenobarbital treatment) of cytochrome P-450 on the vasodilatory action of organic nitrate esters in experimental animals, particularly because induction of cytochrome P-450 was found to increase GTN biotransformation in rat liver microsomes (McDonald and Bennett, 1990).

Considering the above discussion, the formation of 1,2-GDN seems to be more intimately linked to the activation of guanylate cyclase and vascular smooth muscle relaxation than is the formation of 1,3-GDN. This assumption is strengthened by the finding that tolerance to GTN has a much more pronounced inhibitory effect on the formation of 1,2-GDN than on 1,3-GDN (Bennett et al., 1989; Slack et al., 1989). However, the molecular basis for this metabolic selectivity, as well as the relationship between the formation of the two metabolites and nitric oxide and/or nitrosothiols, remains to be investigated; until this has been done, a firm statement cannot be given concerning the relative importance of the different pathways for the pharmacological actions of GTN. Attempts have recently been made to determine the subcellular site for GTN biotransformation in bovine coronary artery in vitro using chemiluminescence detection of the nitric oxide formed (Chung and Fung, 1990). In this study the highest nitric oxide-forming activity was found in the subcellular fractions enriched in marker enzymes for plasma membrane and endoplasmic reticulum and with relatively little nitric oxide formed in the cytosolic fraction. The identity of the GDN metabolites

produced in the different fractions was not determined, and such determinations seem to be particularly important in view of the findings by Lau and Benet (1990) in rabbit liver that microsomal biotransformation of GTN preferentially gave rise to 1,3-GDN.

Little information is known regarding the relationship between selective metabolite formation and pharmacological potency for other organic nitrate esters. However, in one recent study it was shown that ISDN is preferentially denitrated to IS-5-MN in rabbit aorta, with a IS-5-MN/IS-2-MN ratio of about 5 (Slack et al., 1989).

In a recent study, Zimmermann et al. (1991) compared the effect of isobutyl nitrate ester and isobutyl nitrite ester. The nitrite ester induced relaxation in isolated phenylephrine-contracted rabbit aorta about 20-fold more potently. From these results together with the finding that tolerance, and cross-tolerance to other nitrate esters, was much more pronounced for the nitrate ester, the hypothesis was proposed that the nitrate ester is metabolised to nitrosothiols/nitric oxide in the tissue, with formation of the corresponding nitrite ester as an intermediary step.

Another factor that is important to consider when discussing target tissue metabolism of organic nitrate esters in relation to their pharmacological effect is the actual activity of the metabolites. The principal metabolites of both ISDN and GTN are pharmacologically active and induce vascular smooth muscle relaxation, although they are less potent than their respective parent compound when compared under identical experimental conditions (Axelsson et al., 1981; Torfgård et al., 1990a). Concerning the dinitrate metabolites formed from GTN, it is interesting to note that 1,3-GDN appears to be more potent as a blood pressure-lowering agent in human volunteers than 1,2-GDN (Gumbleton and Benet, 1991).

In addition to metabolism of organic nitrate esters within the vascular smooth muscle, it is also apparent that such biotransformation takes place in the blood. Biotransformation of GTN to GDN has been demonstrated in the presence of hemoglobin and also myoglobin (Bennett et al., 1984, 1985, 1986b), and the major metabolite formed was shown to be 1,2-GDN (Bennett et al., 1986b). Biotransformation of GTN to GDN involved oxidation of heme-bound Fe^{2+} to Fe^{3+}, and both carbon monoxide and potassium ferricyanide were able to inhibit the biotransformation of GTN (Bennett et al., 1985, 1986b). Biotransformation of ISDN was also catalysed by hemoglobin, although this reaction seemed to be mostly dependent on SH groups contained in hemoglobin and less dependent on the heme moiety (Bennett et al., 1985).

Thus, important molecular specificity appears to exist regarding the biotransformation of different organic nitrate esters by hemoglobin. In this context it is noteworthy that guanylate cyclase is also a heme protein. However, as of today, there is apparently no information concerning whether or not guanylate cyclase has the capacity to catalyse biotransformation of organic nitrate esters. Experiments aimed at investigating such a biotransforming capacity of guanylate cyclase would be important to perform with purified enzyme in the presence of necessary cofactors, preferably with simultaneous measurement of the formation of metabolites and of nitric oxide or nitrosothiols. Such experiments would give information about the possible function of guanylate cyclase as both a "receptor" and effector molecule for organic nitrate esters. A possible modification of the heme iron ($Fe^{2+} \rightarrow Fe^{3+}$) during biotransformation would also be of importance for tolerance development (see VIII.D).

Biotransformation of GTN to GDN has also been reported to occur in human blood plasma (Fung et al., 1988; Posadas del Rio et al., 1988; Chong and Fung, 1989, 1990). This biotransformation, which is significantly increased by thiols, generates about equal amounts of 1,2-GDN and 1,3-GDN (Chong and Fung, 1990). When different plasma proteins were tested for their ability to degrade GTN, serum albumin was found to display the highest catalytic activity, and the degradation by plasma proteins was markedly higher in the presence of thiols. Thiol-mediated biotransformation of GTN in plasma is also accompanied by formation of S-nitrosothiols (Chong and Fung, 1990), and, therefore, this pathway could theoretically be of importance for the pharmacological effects of organic nitrate esters. However, it seems uncertain to what extent nitric oxide or S-nitrosothiols generated in the blood in vivo can gain access to the vascular smooth muscle cell and activate guanylate cyclase, particularly because it is known that heme proteins are effective inhibitors of nitric oxide stimulation of guanylate cyclase, probably because they bind nitric oxide (Murad et al., 1978). Despite this, the results of Fung et al. (1988) do suggest that at least a portion of the S-nitrosothiol formed in whole blood may be available to activate guanylate cyclase within the smooth muscle cell.

A matter of interest in this context is the possible existence of enantioselectivity regarding the vascular effects of organic nitrate esters. It has been shown that the stereoisomeric forms of isoidide dinitrate showed a 10-fold difference in potency with regard to relaxation and cGMP elevation in isolated rat aorta, with the D-isomer being most potent (Bennett et al., 1986a). With regard to activation of soluble guanylate cyclase, the two stereoisomers were equipotent, and the suggestion was made that the site of enantioselectivity was not the guanylate cyclase itself (Bennett et al., 1988b). In a recent study we found no difference in potency between two enantiomers of butanol-1,2,3,4-tetranitrate (erythritol tetranitrate and racemic threitol tetranitrate) with regard to relaxation of bovine mesenteric artery (Axelsson et al., in preparation; fig. 2). This could indicate that

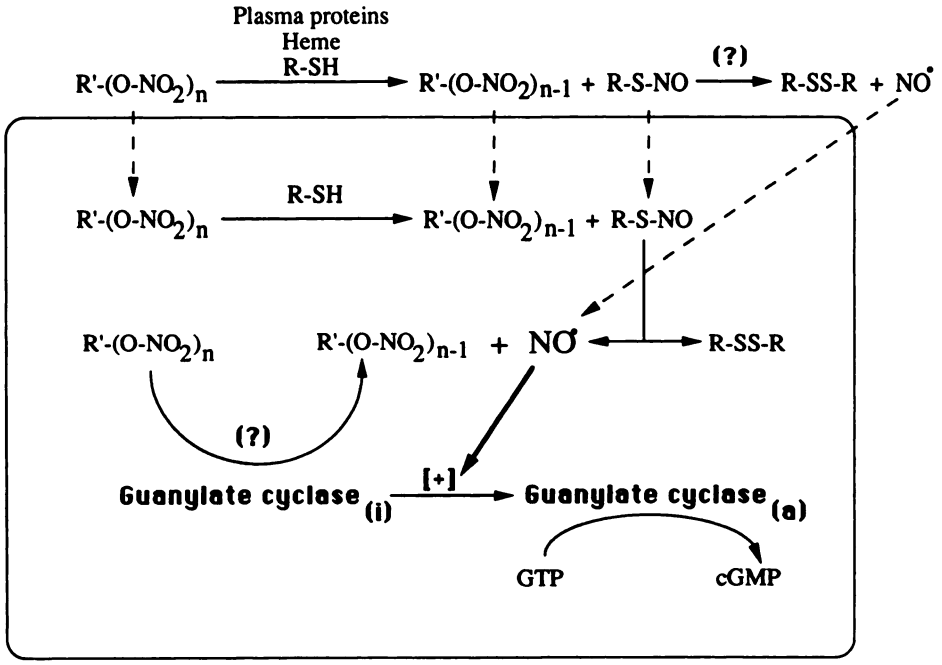

FIG. 7. Metabolic events of possible importance for the vascular smooth muscle relaxant action of organic nitrate esters. In the presence of plasma proteins, heme and thiols organic nitrate esters may be metabolised to nitrosothiols and possibly nitric oxide in the blood. It is suggested that the metabolites, including nitric oxide, may penetrate the plasma membrane and thus gain access to the interior of the smooth muscle cell. Organic nitrate esters may also enter the vascular smooth muscle cell, where it can undergo thiol-requiring metabolism, resulting in nitric oxide which is the activator of soluble guanylate cyclase and cGMP production. In the figure is also shown a hypothetical pathway for nitric oxide formation from organic nitrate esters through metabolism by guanylate cyclase. R-SH, thiol compounds; NO, nitric oxide; R-S-NO, nitrosothiol compound; R'-(O-NO_2)$_n$, organic nitrate ester; R'-(O-NO_2)$_{n-1}$, denitrated organic nitrate ester; R-SS-R, disulfide product; i, inactive enzyme; a, active enzyme.

the enantioselectivity varies between different organic nitrate esters.

Some important aspects of organic nitrate ester biotransformation in relation to their mechanism of action are summarised in fig. 7.

B. Interaction between Organic Nitrate Esters and the Endothelium

The vascular smooth muscle relaxation elicited by organic nitrate esters has been considered to be independent of the endothelium, in contrast to the vasodilatory effect of acetylcholine and various other compounds which are endothelium-dependent vasodilators (see section III.A.1). However, it has gradually become clear that the vascular effect of organic nitrate esters is not endothelium independent but rather endothelium modulated. This may have important implications both when studying organic nitrate esters in vitro and when considering the effect of these drugs in the clinical setting.

We previously showed that GTN-induced relaxation of bovine mesenteric arteries was augmented after removal of the endothelium (Ahlner et al., 1987b). Furthermore, the basal cGMP level was significantly lower in the endothelium-denuded preparations compared with in endothelium-intact preparations (Ahlner et al., 1987b). These results were recently confirmed by other authors (Dinerman et al., 1991; Moncada et al. 1991) and are also in agreement with the findings of Shirasaki and Su (1985) who showed increased vasodilation by SNP and sodium nitrite after endothelial removal. Dinerman et al. (1991) and Moncada et al. (1991) also found that inhibition of endogenous production of nitric oxide with L-arginine analogues potentiated the vasodilator action of GTN and SNP. The underlaying mechanism for this "supersensitivity" to GTN and other nitric oxide producers after endothelial removal is presently unknown. However, the endothelium is known to produce reactive oxidative metabolites that can function as scavengers of nitric oxide, derived from vasodilator drugs (Holland et al., 1990; Sundquist, 1991). Also, the intriguing possibility that the supersensitivity after endothelial removal is due to an upregulation of soluble guanylate cyclase was recently suggested by Moncada et al. (1991). Such a regulation of soluble guanylate cyclase by endogenously produced nitric oxide might have important consequences for the development of tolerance to organic nitrate esters as discussed in section VIII.

V. Pharmacokinetics of Organic Nitrate Esters

A. Pharmacokinetic Studies of GTN Factors to Consider

Because of its physical and chemical properties (see above), and hence pharmacokinetic behaviour, a number of things have to be considered when conducting and interpreting pharmacokinetic studies of GTN.

1. Sorption of glyceryl trinitrate to materials. It has

long been known that sublingual tablets containing GTN lose potency over time. This seems not to be due to chemical degradation but to physical volatilisation with subsequent sorption to various materials in contact with the tablets (Banes, 1968; Fusari, 1973; Dorsch and Shangraw, 1975; Pikal et al., 1976, 1977). The problem with sorption to materials has also long been recognised by pharmacists in connection with storage of GTN solutions in plastic containers (Boylan et al., 1978; Crouthamel et al., 1978; McNiff et al., 1979; Aman et al., 1980; Christiansen et al., 1980; Roberts et al., 1980) and by physicians administering i.v. GTN through plastic tubing (Cossum et al., 1978; Baaske et al., 1980; Roberts et al., 1980; Elliott and Quinn, 1982; Cooke et al., 1985). Losses to materials in cardiopulmonary bypass apparatus, mainly to the polyurethane-defoaming sponge in the oxygenator, have been described by Dasta et al. (1983). In a later study, Dasta et al. (1986) reported that cardiopulmonary bypass increased GTN clearance by 20% in patients receiving an i.v. infusion of GTN.

In particular, bags and infusion sets made of PVC constitute a practical problem. The loss in PVC bags seems to be entirely due to retention of GTN in the plastic, not to degradation (McNiff et al., 1979; Roberts et al., 1980; Yacobi et al., 1983).

De Rudder et al. (1987) studied the sorption of GTN to different infusion sets and burettes under simulated perfusion conditions, with a droplet rate corresponding to clinically relevant infusion rates (62.5 and 12.5 μg/min). Bags made of PVC showed a pronounced sorption of GTN. For three different PVC administration sets, the sorption of GTN was also high, and only 50% or less of the expected amount of GTN was delivered out of the infusion sets. Attempts to presaturate the sets by rinsing them with the glucose/GTN solution for 10 min at a flow rate of 3 ml/min were evaluated. This procedure had only a minor influence on the recovery of drug, and the authors stated: "It is obvious that concentrations of GTN in the effluent of PVC-giving sets, will never reach the original concentration under normal infusion conditions." This is in accordance with an earlier study by Nix et al. (1984).

Leor et al. (1989) found that the use of a 5% ethanol solution led to a significant decrease in drop volume and a concomitant increase in the number of drops per unit volume. The surface tension-lowering effect might account for decreases of ≥25% in delivered GTN, when drop-counting infusion pumps are used. Recently, Loucas et al. (1990) showed that the ionic strength of the vehicle was of importance for the sorption of GTN to PVC infusion sets. Unfortunately, the choice of material (PVC) and the rapid infusion rate used (667 μg/min) diminish the practical clinical importance of the report, although it may be of theoretical interest.

In conclusion, in pharmacokinetic and hemodynamic studies of GTN involving i.v. administration, it is of utmost importance that the materials in bags and infusions sets are inert to GTN. The same is true in clinical practice: i.v. administration of a potent drug, such as GTN, to severely ill patients can hardly be justified if it is not known how much of the given drug will actually reach the patient. Today, infusions sets that resist sorption of GTN are available.

It is important to consider the material even during in vitro studies (Ahlner et al., 1987c) and to be aware of the cross-contamination of GTN into the pipet, when using a pipet with exchangeable tips (Torfgård et al., 1990b), when analysing GTN.

2. Analysis of glyceryl trinitrate. The first method for determining low concentrations of GTN in plasma was published by Rosseel and Bogaert in 1973. They used GC together with ECD to achieve a sensitivity of 0.5 ng/ml using a 5-ml plasma sample. The method had several disadvantages: a relatively large sample volume was required, the GC traces were excessively affected by interference peaks, and a total analysis time of 90 min per sample was required. Yap et al. (1978) modified the method and made it more selective for GTN and also obtained cleaner chromatograms; however, no less than 12 extractions had to be used. This method and others based on the same principle were time consuming, the level of variance was high, and the metabolites were not determined simultaneously.

A useful GC variant is the application of capillary columns. Noonan et al. (1984) developed a specific and sensitive capillary GC assay using the on-column injection technique. This method, which is capable of analysing picogram concentrations of GTN in human plasma, uses a double extraction with pentane, and the assay limit of quantitation is 25 pg/ml.

The introduction of combined GC and mass spectrometry was a major advance in specificity, although the instrumentation is expensive. Gerardin et al. (1982) presented a GC-mass spectrometric method using ^{15}GTN as the internal standard; this method allows estimation of concentrations in plasma as low as 0.2 nmol/liter (50 pg/ml). Jaeger et al. (1987) developed a GC-mass spectrometric method for the determination of GTN and its dinitrate metabolites. However, with this method it is not possible to analyse GTN and its metabolites in one chromatogram. The lower limit of quantitation for GTN is reported to be 6 pg/ml. The method shows a high degree of accuracy but the imprecision is up to 18% in the lowest analytical region. The authors use capillary GC with ECD for the routine analysis of the GDN metabolites, mostly because mass spectrometry is an expensive method. They claim lower limits of quantitation of 0.25 and 0.1 ng/ml for 1,2-GDN and 1,3-GDN, respectively.

There are methods available for determination of GTN, as well as its dinitrate metabolites, based on GC-ECD (Carlin et al., 1988; Langseth-Manrique et al., 1986;

Lee et al., 1988; Svobodova et al., 1988). All of these methods include only a single extraction step, and all use capillary GC with ECD. The most sensitive method is the one presented by Lee et al. (1988), with plasma detection limits of 0.025, 0.1, and 0.1 ng/ml for GTN, 1,2-GDN, and 1,3-GDN, respectively. Recently, a more rapid method for the simultaneous quantitation of GTN and its dinitrate metabolites was developed using capillary GC with ECD (Torfgård et al., 1990b). This method includes only one extraction step, using a dichloromethane-pentane mixture, and is suitable for a large number of samples. A more extensive discussion of the technical problems of the analytical methods are not within the scope of this review. Regarding methods suitable for pharmacokinetic studies, today it is possible to use GC-ECD methods; it is not necessary to use mass spectroscopy. In pharmacokinetic and hemodynamic studies, it is important to measure the metabolites, at least the dinitrates, simultaneously. A summary of some of the methods is given in table 2.

3. Handling of glyceryl trinitrate samples. A number of studies have shown that GTN is lost from incubated human blood (Lee, 1973; Noonan and Benet, 1982; Sokoloski et al., 1983), resuspended erythrocytes (Armstrong et al., 1980a; Wu et al., 1981), and plasma (Maier et al., 1979; Armstrong et al., 1980a; Noonan and Benet, 1982; Bennett et al., 1984). In a study by Cossum and Roberts (1985) it was found that, in an erythrocyte suspension, therapeutic concentrations of GTN (0.8 to 10 ng/ml) were rapidly metabolised (half-life 3 min) to dinitrate metabolites and subsequently to mononitrates. The metabolism of GTN and its metabolites was concentration dependent, and the metabolism of GTN by erythrocytes was partially inhibited by the presence of metabolites. The metabolism of GTN in blood samples collected from patients could be stopped by adding the collected blood to chilled tubes containing the enzyme inhibitor iodoacetamide. The half-life of GTN in blood at 2°C was about 27 min.

For practical purposes, it is important that the sample is handled at 0 to 4°C and assayed as quickly as possible (Noonan et al., 1984). The blood sample has to be drawn into chilled tubes (glass) and subjected to immediate centrifugation. The plasma should be frozen immediately in a dry ice bath. The total time required to withdraw the blood sample and centrifuge to obtain plasma should be <2 min (Noonan et al., 1984). The same authors have also addressed the question of stability of the frozen samples. They found that GTN was stable in plasma when stored at −20°C for a minimum of 60 days. In a recent study by the same group, samples were stored at −80°C until assayed (Nakashima et al., 1990). According to Maier et al. (1979), significant drug degradation, i.e., a 10% loss in 40 days, occurs when human plasma samples containing GTN are stored at −20°C.

4. Influence of sampling site on concentrations of glyc-

TABLE 2
Analytical methods for GC determination of GTN

Method (reference)	Sample size (ml)	Extraction	Detection	Lower limit of determination (GTN)	Time (GTN) (min)	Total time (min)	GDN simultaneously
Rosell and Bogaert, 1973	5.0	3 × ethyl acetate	ECD	0.5 ng/ml	10	≈90	No (ISDN)
Yap et al., 1979	0.2	12 × hexane	ECD	0.1 ng/ml	6	≈15	No
Gerardin et al., 1982	1.0	1 × pentane/methyl acetate	Mass spectrometry	50 pg/ml	2	≈3	No
Noonan et al., 1984	1.0	2 × pentane	ECD	25 pg/ml	8.6	≈13	No
Langeseth-Manrique et al., 1986	2.0	1 × benzene	ECD	≈11 pg/ml	10.5	≈14	Yes
Jaeger et al., 1987	2.0	1 × pentane	Mass spectrometry	6 pg/ml	Not given	Not given	No
Jaeger et al., 1987	2.0	1 × dichloromethane	ECD				Only GDN
Carlin et al., 1988	1.0	1 × methyl t-butyl ether	ECD	10 ng/ml	9.4	≈10	Yes
Lee et al., 1988	1.0	1 × methylene chloride/pentane	ECD	25 pg/ml	Not given	≈22	Yes
Svobadova et al., 1988	2.0	1 × pentane/acetyl acetate	ECD	0.1 ng/ml	≈11	≈28	Yes
Torfgård et al., 1990	1.0	1 × dichloromethane/pentane	ECD	0.1 ng/ml	5.2	≈9	Yes

eryl trinitrate in plasma. Armstrong et al. (1980b) noted that, when using arterial blood samples, plasma concentrations of GTN were higher than previously reported plasma concentrations in venous samples (Wei and Reid, 1979). Hill et al. (1981) measured plasma concentrations from different sampling sites after administration of GTN (0.8 mg) via the intranasal route. Samples were collected from a radial artery, from the pulmonary artery via the internal jugular vein, and from a peripheral vein. For at least the first 8 min after the administration of the dose, the GTN concentrations were different in the blood obtained via these three blood vessels. Concentrations were highest in the central venous plasma (11 ng/ml), intermediate in arterial plasma (~5 ng/ml), and lowest in peripheral venous plasma (~2 ng/ml). The influence of different sampling sites on plasma concentrations was systematically studied by Armstrong et al. (1982). Twenty patients with chronic congestive heart failure underwent hemodynamic monitoring during i.v. GTN infusion. The end point was a reduction in PCWP by at least 25% of its control value. The concentrations of GTN in the pulmonary artery and systemic artery were similar (mean ± SD, 29.8 ± 52.8 and 25.1 ± 48.4 ng/ml, respectively) and considerably higher than the concentration in the peripheral vein (7.3 ± 15.4 ng/ml). There was 17.4 ± 19.1% extraction of GTN across the pulmonary vascular bed and 60.8 ± 27.2% extraction across the arterial-venous bed. Fung et al. (1986) studied the pharmacokinetics of GTN in rats, and in accordance with the studies mentioned above considerably higher concentrations of GTN were found in arterial plasma compared with venous plasma. Fung et al. (1986) also showed that GTN pharmacokinetics are dependent on cardiac output and thus provided a hemodynamic explanation for the high variability in GTN plasma concentrations.

Gjesdal et al. (1985) studied gun powder production workers and reported enormous differences between the plasma concentrations of GTN in blood samples obtained from a cubital vein and those from the femoral vein; in one individual a value of 1015 nmol/liter was measured in the cubital vein and 11 nmol/liter in the femoral vein. The explanation for this is that the hands and arms of the workers were probably heavily exposed to GTN.

5. Influence of route of administration. Noonan and Benet (1987) proposed that the route of administration is important for the formation of metabolites of GTN. Plasma concentrations of 1,2- and 1,3-GDN were measured in four healthy volunteers receiving i.v. infusions (10, 20, and 40 μg/min), topical ointment (20 mg/200 cm^2), and an oral solution (6.5 mg) of GTN and in two subjects who received GTN sublingually (0.4 mg). The ratio of 1,2-GDN to 1,3-GTN (dinitrate ratio) was determined and was found to be 7.36, 4.60, 3.86, and 1.99 for i.v., sublingual, topical, and oral doses, respectively. The authors speculated that different metabolic specificity of enzymes may be present in the gut, liver, skin, sublingual mucosa, and blood vessels. However, recently, the same group of researchers (Nakashima et al., 1990) found in six volunteers who were given six different doses (0.4 to 13 mg) of an oral GTN solution that the dinitrate ratio seems to be dose dependent. The dinitrate ratio for the 0.4-mg dose was significantly different when compared with doses of ≥1.6 mg. The ratios of metabolites for 0.4-mg doses of GTN were similar for oral and sublingual administration as reported in a previous study by the research group (Noonan and Benet, 1987). In this study, a large intrasubject and intersubject variability was also noted for the metabolism and dinitrate ratio.

6. Influence of posture. Curry and Kwon (1985) reported that plasma concentrations of GTN were influenced by posture. They administered 0.4 mg of GTN sublingually to a group of six subjects sitting and to another group in the supine position. The mean maximum concentration in the supine group was three to four times higher than that in the sitting group, and the mean AUC of plasma concentration versus time was also approximately three times higher for the group in the supine position. However, Heidemann et al. (1987) found no difference when comparing, in a randomised crossover fashion, the application of a transdermal GTN plaster (releasing 10 mg GTN during 24 h) to eight healthy subjects either in a standing/ambulant or a supine position. The plasma concentration of GTN was evaluated for 12 h, and the mean GTN plasma concentration for six time points between 2 and 12 h after the dose ranged from 0.85 to 1.80 nmol/liter for subjects in the supine position and from 0.52 to 1.45 nmol/liter for the standing/ambulant position. No significant difference in AUC was found between transdermal GTN applied in the supine position and in the standing/ambulant position. Recently, Lefebvre et al. (1990) showed that, in nine healthy subjects treated with a transdermal GTN plaster (10 mg/24 h), the average plasma concentration almost doubled in the sitting group compared with the supine group. These results are quite different from those of Curry and Kwon (1985), but the reasons for the differences are not known. However, it does seem to be important to standardise the position of the subjects when obtaining plasma samples for pharmacokinetic studies.

7. Effect of exercise on the pharmacokinetics of glyceryl trinitrate. This question has been the subject of three studies. Barkve et al. (1986) showed that the peak plasma concentration of GTN administered as a transdermal patch was three times higher in subjects who exercised for 20 min than in nonexercising subjects. A weak correlation between skin temperature and plasma concentration of GTN was found. This was interpreted as an increased percutaneous absorption of GTN due to an increased cutaneous blood flow (van Baak, 1990). On the other hand, Weber et al. (1987) demonstrated that the

increase in plasma concentration of GTN during exercise was not significantly different after transdermal and i.v. administration. After both formulations, a significant increase in plasma GTN was seen during exercise as compared with during rest and recovery. Weber et al. (1987) suggested that the increased concentration during exercise was due to the exercise-induced reduction of hepatic blood flow and thus of GTN clearance. Lefebvre et al. (1990) studied the effect that exercise on a bicycle had on the plasma concentration of GTN in nine healthy subjects treated with a transdermal GTN patch (10 mg/24 h). They found a marked increase in plasma concentration compared with a supine resting position and even compared with sitting. The maximum plasma concentration was reached 5 min postexercise.

In conclusion, pharmacokinetic studies of organic nitrate esters should include estimation of active metabolites. Studies must be conducted under standardised conditions regarding posture, exercise, food intake, sampling site, etc. Studies should be performed on the actual patient group. The influence of concomitant medication has to be considered. Relatively little is known about the effect of multiple dosing; studies of patients receiving chronic medication should be performed. The handling of samples is also important, as is the material used in the infusion sets.

B. Pharmacokinetic Data concerning Glyceryl Trinitrate

1. Absorption and bioavailability. GTN is readily absorbed after sublingual and transdermal administration and, according to experimental studies of rats, also from the gastrointestinal tract (Hodgson and Lee, 1975). The bioavailability after oral administration, however, is very low because of an extensive first-pass metabolism in the liver and possibly in the gut wall. In some studies, GTN was not detected in the systemic circulation after oral administration of the substance, whereas high concentrations of metabolites were found (Noonan and Benet, 1986; Ochs et al., 1985; Laufen and Leitold, 1988). Noonan and Benet (1986) investigated an oral solution of GTN (6.5 mg) ; the detection limit of the assay was 25 pg/ml. Levels of both 1,2-GDN and 1,3-GDN were measured in four subjects receiving the solution. The plasma concentrations of the 1,2-GDN metabolite were always higher than those of the 1,3-GDN metabolite, and the absolute (peak) concentrations were in the range of 8 to 15 ng/ml for 1,3-GDN and 15 to 30 ng/ml for 1,2-GDN. Ochs and coworkers (1985) studied a sustained-release preparation (18 mg), and they used an assay procedure sensitive down to 50 pg/ml. Only 1,2-GDN was measured, and the concentration was approximately 5 to 10 ng/ml. There were a number of earlier studies showing measurable levels of GTN (Blumenthal et al., 1977; Bashir et al., 1982) after oral administration. The reason for the discrepancies are not known, although the specificity of the methods used might be questioned. The newly developed methods that can simultaneously measure GTN and the dinitrate metabolites should be used in pharmacokinetic studies.

Porchet and Bircher (1982) compared pharmacodynamic activity as measured by digital plethysmography, a method previously accepted by the United States Food and Drug Administration for determination of bioavailability of organic nitrates, after i.v. infusion (8 and 25 μg/min) and oral administration (0.8 mg) of GTN. These authors found an oral bioavailability of 2 ± 4% (mean ± SD) in normal volunteers. A similar approach was taken by Nyberg and Westling (1981). The effects of GTN (0.5 mg sublingually and 6.5 mg orally) and a placebo tablet on heart rate and blood pressure during standing, and on plethysmographic arterial pulsation in the calf, were studied for up to 8 h. Sublingual GTN increased heart rate and arterial pulsation. Oral GTN had no effect on heart rate but did increase pulsations as compared to placebo. In the orthostatic test, heart rate and pulse amplitude were affected by both forms of administration. When the the AUC for these variables were compared, it was calculated that, in comparison with sublingual GTN, about one-third of the oral GTN was biologically effective during 8 h; this was taken as an indication of a "bioavailability" of approximately 30% for the oral preparation. A large interindividual variation was noted.

The bioavailability after sublingual administration was studied by Noonan and Benet (1985). They studied eight healthy volunteers who were given sublingual GTN tablets (0.4 mg). The absolute bioavailability was found to be 36.2 ± 24.9% (mean ± SD). The bioavailability varied widely from 2.6 to 113%, as did the time to peak concentration. Even within the same individual, there were extreme variations between different occasions of administration. Noonan and Benet (1987) only studied the plasma concentrations of the dinitrate metabolites after sublingual administration in two individuals. They found the 1,2-GDN/1,3-GDN ratio to be 4.6, compared with 1.99 after oral administration (6.5 mg).

The bioavailability after transdermal administration has been estimated to be approximately 70% (Nakashima et al., 1987). This is in agreement with a study of healthy volunteers in which Imhof et al. (1984) compared plasma concentrations of GTN after patches and after i.v. infusions and estimated a bioavailability of approximately 75% for the transdermal administration. Comparatively, in a study on rhesus monkeys Wester and coworkers (1983) found an absolute bioavailability of 57%. Noonan and Benet (1987) measured the plasma concentrations of the dinitrate metabolites after transdermal administration of GTN and found the ratio of 1,2-GDN/1,3-GDN to be 3.86.

2. Distribution. The distribution of GTN has not been widely studied. Varying values for apparent volume of distribution (a derived parameter) have been given, which is not surprising, because the studies have been performed under different conditions and have used dif-

ferent assay procedures. According to McNiff et al. (1981), apparent volume of distribution is approximately 3 liters/kg and the calculated amount of GTN residing in the plasma compartment should be approximately 1%. This estimate was based on a study in which 0.51 ± 0.06 mg was infused during 32 min in nine normal volunteers.

In distribution experiments in rats, Hodgson and Lee (1975) found concentrations of total radioactivity that were 4.4 and 7.8 times higher in the liver and 2.4 and 2.8 times higher in the kidney than in plasma, 4 and 24 h, respectively, after oral administration of [^{14}C]GTN. According to other studies performed on rats (Fung et al., 1984) and sheep (Cossum et al., 1986), GTN is taken up by the vasculature, where it is extensively metabolised. Fung et al. (1984) found that GTN concentrations in blood vessels (ng/g) were generally higher (10-fold) and declined about twice as slowly as GTN plasma concentrations (ng/ml). The conclusion was that vascular (and other) extrahepatic tissue can take up and/or metabolise organic nitrates.

The tissue distribution of GTN was recently studied in the rat (Torfgård et al., 1989). When 50 mg/kg was given subcutaneously, the highest concentration was found in adipose tissue. Levels of GTN were found to be 40 to 150 times higher in adipose tissue than in plasma 1 to 24 h, respectively, after the subcutaneous injection. The concentrations of GTN in brain, heart, and aortic tissue were 2- to 3-fold that in plasma. Such an accumulation (concentration) in adipose tissue might well have pharmacokinetic and clinical implications, although this has not been studied in humans.

Recently, results of an autoradiographic study of the distribution of ^{14}C-labeled GTN in mice was reported (Torfgård et al., 1990c). A rather homogeneous distribution of radioactivity was shown. High levels were found in the nasal and tracheobronchial mucosa and in the brown fat. Radioactivity was also found in the urine and in the intestinal content, indicating a rapid excretion of GTN and/or its metabolites. The level of radioactivity in the nasal and tracheobronchial mucosa was markedly decreased in mice pretreated with metyrapone, a finding that suggests the involvement of the cytochrome P-450 mixed oxygenase system in the formation of GTN metabolites in the mucosal linings of the respiratory tract.

3. Metabolism and elimination. For a long time, the liver, because of its glutathione S-transferase activity, was considered to be the predominant site of metabolic breakdown of GTN (for review, see Needleman, 1975). However, the high clearance values for GTN, far exceeding the hepatic blood flow and even cardiac output, indicate a considerable extrahepatic metabolism. Recently, Posadas del Rio et al. (1988) demonstrated in vitro that the small intestinal mucosa metabolises organic nitrates more rapidly than do the liver and kidney. A rapid degradation of GTN has been shown to occur in red blood cells (Armstrong et al., 1980a; Noonan and Benet, 1982), and glutathione S-transferase has previously been isolated from these cells (Marcus et al., 1978). In addition, it is now well-known that accumulation and degradation of GTN occur in the vessel walls (Fung et al., 1984). Recently, soluble glutathione S-transferase activity in different tissues of the rat was shown to parallel the metabolism of GTN (Taylor et al., 1989). Liver was the most active tissue, but activity was also present in the heart, kidney, and gut. Very recently, three and four different forms of glutathione transferase with an activity toward GTN were purified and characterised from human aorta and from human heart, respectively (Tsuchida et al., 1990). Metabolism of GTN, generating nitric oxide or some related molecule, has been implicated as a prerequisite for activation of the soluble guanylate cyclase (Bennett et al., 1989), and it has been shown that the metabolism occurred concurrently with vascular smooth muscle relaxation (Brien et al., 1986).

In a study by Kawamoto et al. (1987), it was confirmed that biotransformation of GTN occurred concomitantly with relaxation of bovine pulmonary artery and vein. However, the concentration of GDN in the vein was significantly less than in the artery, even though relaxation was much greater in the former. A simple linear relationship between GTN biotransformation and relaxation is not apparent. Slack et al. (1989) showed that isolated rabbit aortic strips made tolerant in vitro to GTN showed an impaired relaxation to GTN and had metabolite concentrations that were significantly lower than in nontolerant tissue. This supports the hypothesis that organic nitrate biotransformation is required for organic nitrate ester-induced vasodilation to occur. The subcellular location of the metabolic activation process of GTN in vascular smooth muscle has not been identified. Kawamoto et al. (1988) found no specific binding of [^{3}H]GTN to subcellular fractions of vascular smooth muscle cells from the bovine pulmonary vein. However, Chung and Fung (1990) studied the metabolic activation of GTN to nitric oxide in subcellular fractions of vascular smooth muscle cells from bovine coronary arteries and found that the enzyme(s) responsible for the activation of GTN is particularly associated with the plasma membrane.

Servent et al. (1989) recently showed that reductive denitration of GTN by liver microsomes is catalysed by cytochrome P-450. These authors claim that this is the main metabolic transformation of GTN by liver microsomes and that it is distinct from the previously described transformation that involves glutathione S-transferases (Needleman and Hunter, 1976; Taylor et al., 1989). Bennett and coworkers (1990) also provided strong evidence for the involvement of cytochrome P-450 in the biotransformation of organic nitrates by rat hepatic microsomes, but so far there is no evidence that cytochrome P-450 is

involved in the biotransformation of GTN in the vascular smooth muscle, because this has not been studied.

The clearance of GTN in humans seems to be subject to huge interindividual variations. Noonan and coworkers (1985) presented values between 5.5 and 71 liters/min after i.v. infusion (10 to 40 μg/min) in healthy volunteers. Clearance was not directly related to plasma concentrations but was found to decrease to a constant value (11 ± 6 liters/min, mean ± SD) as GTN concentrations initially increased. Hysteresis was observed in the dose versus steady-state concentration curve, and the plasma half-life in the study by Noonan et al. (1985) was 2.3 min. After i.v. administration (73 to 440 μg/min), Armstrong et al. (1980b) estimated the half-life to be 1.9 min, whereas after sublingual administration (0.6 mg) a half-life of 4.2 min was found by the same group (Armstrong et al., 1979). McNiff et al. (1981) reported a half-life of 2.8 min after i.v. administration (0.6 mg; rate 18 μg/min) and determined clearance values of 29.8 to 78.3 liters/min. Armstrong et al. (1979) reported a clearance of 28 liters/min after sublingual administration to normal subjects. In patients with congestive heart failure, somewhat lower figures were observed (Armstrong et al., 1980b). The more advanced the venous congestion, the lower the total body clearance seemed to be. In less severe cases, the clearance was 13.8 ± 5.8 liters/min, whereas in patients with severe congestive right heart failure it was only 3.6 ± 1.8 liters/min. Dose-dependent pharmacokinetics of GTN have been observed in humans (Noonan et al., 1985) and in dogs (Lee, 1987).

The terminal half-lives of the dinitrate metabolites are longer than that of the parent compound. In dogs a half-life of 40.6 min was reported for 1,2-GDN and 48.5 min for 1,3-GDN, when GTN was administered intravenously (6 μg/kg/min) (Miyazaki et al., 1982), which is consistent with the findings of Lee (1987). Lee studied the half-lives of 1,2-GDN and 1,3-GDN, both when a high (0.25 mg/kg) and a low (0.025 mg/kg) i.v. dose of GTN were given. For the high dose, the half-lives in plasma were 50 and 47 min for 1,2-GDN and 1,3-GDN, respectively; for the low dose, the half-lives were 38 and 39 min, respectively. After oral administration (6.5 mg) in humans, Noonan and Benet (1987) reported that the apparent terminal half-lives in plasma were 44.1 ± 8.3 and 42.6 ± 10.6 (mean ± SD) min for 1,2- and 1,3-GDNs, respectively. Corresponding figures after transdermal administration (20 mg over 200 cm^2) were 35.8 ± 10.3 and 67.3 ± 11.7 min for the 1,2- and 1,3-dinitrate metabolites of GTN, and after sublingual administration (0.4 mg) 36.5 ± 10.3 and 43.8 ± 12.0 min, respectively. After i.v. infusion (10 to 40 μg/min), values of 33.2 ± 6.5 and 57.2 ± 30.0 min were given.

In conclusion, the pharmacokinetics of GTN are dose dependent. The plasma half-life is very short (5 to 6 min), the substance is extensively metabolised, and the bioavailability after oral administration is extremely low (1 to 2%). The metabolites formed are pharmacologically active, and they have longer plasma half-lives (40 to 60 min).

C. Pharmacokinetic Data for Isosorbide Dinitrate and Isosorbide-5-Mononitrate

1. Sorption to materials. Several reports have dealt with the absorption of ISDN by i.v. delivery systems (Cossum and Roberts, 1981; Lee and Fenton-May, 1981; Remon and Bogaert, 1983; Lee, 1986; De Muynck et al., 1988). The latter paper revealed that, when infusion sets made of PVC are used, there is an approximately 50% loss of ISDN, whereas if polybutadiene is used, the loss is negligible. It must be concluded that, if an infusion of ISDN is to be given, special tubing has to be used.

2. Analysis of isosorbide dinitrate and isosorbide-5-mononitrate. Because both metabolites of ISDN (IS-5-MN and IS-2-MN) are pharmacologically active, pharmacokinetic studies must include measurement of these metabolites.

Several GC methods for the determination of ISDN and its active metabolites have been published (Rosseel and Bogaert, 1979; Straehl and Galeazzi, 1985; Jaeger et al., 1987). In the method developed by Jaeger et al. (1987), GC with an electron capture detector was used. The lower limits of quantitation were reported to be 0.5, 1, and 5 ng/ml, respectively, for ISDN, IS-2-MN, and IS-5-MN.

Morrison and Fung (1984) used a gas-liquid chromatographic method with ECD and reported a limit of quantitation of approximately 5 ng/ml for the mononitrates and 1 ng/ml for ISDN.

Straehl and Galeazzi (1985) used two separate gas-liquid chromatography methods. For the quantification of unchanged ISDN, a modification of the method described by Laufen et al. (1978) was used. The detection limit for ISDN was approximately 0.5 ng/ml. The detection limits for IS-5-MN and IS-2-MN were 0.5 and 1 ng/ml, respectively. Abshagen et al. (1985) used a GC method with ECD for determination of ISDN, IS-5-MN, and IS-2-MN. The detection limits reported were 2, 5, and 3 ng/ml for ISDN, IS-5-MN, and IS-2-MN, respectively.

In general the same methods that have been mentioned for ISDN have also been successfully applied to the analysis of IS-5-MN. Straehl and Galeazzi (1984) described an electron capture GC method for IS-5-MN, using a capillary column and with ISDN as the internal standard; the lower limit of detection was 1 ng/ml of plasma.

3. Absorption and bioavailability. After oral administration of ISDN, complete absorption occurs from the gastrointestinal tract of rats (Reed et al., 1977) and humans (Down et al., 1974; Chausseaud et al., 1975). However, the absolute bioavailability after oral administration has been estimated to be only 19 to 29% due to first-pass metabolism (Taylor et al., 1982; Abshagen et al., 1985).

Considerable interindividual variability seems to exist; after the same dose in different individuals, 5- to 11-fold variations in peak concentrations have been reported (Thadani et al., 1980, 1981, 1982).

The absolute bioavailability of ISDN after sublingual administration amounts to 30 to 58% (Taylor et al., 1982; Abshagen et al., 1985; Morrison et al., 1983). Following topical administration, bioavailability has been reported to range from 10 to 30% (Morrison et al., 1983).

After oral administration there is a nearly complete absorption and no first-pass metabolism for IS-5-MN and hence a bioavailability of almost 100% (Abshagen and Spörl-Radun, 1981; Major et al., 1984; Taylor et al., 1981). The rate of absorption of IS-5-MN is slowed by food ingestion, but overall bioavailability is unchanged (Laufen and Leitold, 1984).

4. Distribution. The apparent volume of distribution for ISDN has been estimated to be approximately 100 liters in humans (Morrison et al., 1983; Straehl and Galeazzi, 1985).

In animal experiments, high concentrations of ISDN have been shown in vessel walls. Reed et al. (1977) administered ISDN orally (2.0 to 2.2 mg/kg) and intravenously (0.8 to 1.1 mg/kg) to rats and found 40-fold higher drug concentrations in the wall of the inferior vena cava than in plasma. Fung et al. (1984) reported that, after i.v. infusion of ISDN (10.5 μg/min for 1 h) in the rat, ISDN levels in vessel walls near the infusion site were 15-fold higher than in plasma.

Schneider et al. (1990) studied the concentration of ISDN, IS-5-MN, and IS-2-MN in plasma, saphenous vein wall, and pectoral muscle from eight patients undergoing coronary bypass surgery. The patients were treated for 2 days with ISDN, 240 mg/day. The plasma and tissue samples were obtained during the operation, 10 to 12 h after the last dose. IS-2-MN and IS-5-MN were present in plasma and tissues in the same concentrations. Mean ISDN concentrations in tissues were considerably higher than in plasma; the molar concentration ratio of saphenous vein wall/plasma was 7.21. Hence, there seems to be an accumulation of ISDN in the vessel walls.

After i.v. infusion of 20 mg IS-5-MN to healthy volunteers, an apparent volume of distribution of 0.62 liter/kg was noted (Abshagen and Spörl-Radun, 1981); this value is considerably less than those for GTN and ISDN.

5. Metabolism and elimination. ISDN is degraded metabolically by denitration and glucuronidation to IS-2-MN and IS-5-MN and further to the glucuronides and sorbitol. The plasma half-life for ISDN in humans has been estimated to be about 1 h (Abshagen et al., 1985; Taylor et al., 1982). After chronic oral dosing, a slow terminal half-life of elimination of 7.7 ± 2.6 (mean ± SD) h has been reported (Fung and Parker, 1983). According to studies in which ^{14}C-labeled ISDN was used, approximately 60% of the metabolism of ISDN after oral administration proceeds via IS-5-MN and about 25% via IS-2-MN (Down et al., 1974). Chronic oral dosing of ISDN at a high level (720 mg/day) resulted in prolonged high plasma concentrations of ISDN, as well as higher levels of the metabolites in plasma (as much as 5-fold for IS-2-MN and 30-fold for IS-5-MN) (Shane et al., 1978). The same observations were made by Bruyneel et al. (1982).

Morrison and Fung (1984) studied the partitioning and metabolism of ISDN in human and rat blood. At 37°C in human blood, they found that the apparent in vitro partitioning ratio of erythrocytes/plasma was 0.13. In spite of the poor affinity for red blood cells, ISDN degradation in whole blood was mediated primarily via this blood fraction. Loss of ISDN in blood appeared to proceed exclusively through its mononitrate metabolites, resulting in a 6:1 product ratio for the 5-mononitrate to its 2-isomer. The half-life of ISDN in human blood was found to be about 100 min. However, the degradation of ISDN in erythrocytes contributes very little to the total body clearance of the drug, probably <1%.

IS-5-MN is metabolised by glucuronidation to inactive metabolites. Only 2% of IS-5-MN administered orally is excreted unchanged in urine (Abshagen, 1987). A clearance of 115 ml/min was calculated, and the plasma half-life has been estimated to be 4.2 to 4.6 h (Major et al., 1984; Straehl et al., 1984; Abshagen and Spörl-Radun, 1981).

The kinetics seem to be dose linear (Abshagen 1987), and this applies to multiple dosing as well (Mannebach et al., 1981), in contrast to ISDN (Bogaert and Rosseel, 1981; Bruyneel et al., 1982; Fung. et al., 1981; Taylor et al., 1978). In the study by Mannebach et al. (1981), 18 patients with coronary artery disease were given 20 mg IS-5-MN orally three times daily for 1 week. The resulting AUCs within one dosage interval during steady-state were not different from the total AUCs after a single oral dose of 20 mg in normal volunteers.

In conclusion, ISDN is almost completely absorbed when given orally. It is metabolised to IS-5-MN and IS-2-MN. The bioavailability is approximately 20%, and the plasma half-life is approximately 1 h. IS-5-MN is the principal metabolite, and it is pharmacologically active. Given orally IS-5-MN has an almost complete bioavailability. The plasma half-life is 4 to 5 h, and its metabolites are inactive.

D. Relationship between Pharmacokinetics and Therapeutic Effect of Nitrates

It is difficult or impossible to describe the therapeutic effect of nitrates as a dose-response or concentration-response relationship. The plasma concentration of nitrates is not directly related to the therapeutic effect. This observation may be due to several factors:

1. The therapeutic action of nitrates is a secondary effect, resulting from different effects in various segments of the circulation (and perhaps also from effects

on platelets and myocardium). In each segment there is a different concentration-response curve. Moreover, the relative contribution of each action (coronary dilation, arterial dilation, and venodilation) is variable and differs in various patients and conditions (Hollenberg and Go, 1984).

2. Nitrates are metabolised in the vascular wall, and this process is associated with the mechanism of action of the drugs. It has been proposed that the pharmacodynamic effects of nitrates are better correlated with vascular wall concentrations than with plasma concentrations (Fung et al., 1984).

3. The sensitivity of vascular walls to nitrates shows a marked intersubject variability (Eichler et al., 1987).

4. Part of the pharmacodynamic effect is exerted by metabolites. This is certainly true for ISDN and, therefore, much of the activity of this compound is due to the formation of active metabolites, especially IS-5-MN. Some of the therapeutic effect of GTN may also be dependent on the dinitrate metabolites of the drug, although this has not been extensively studied.

5. The concentration-response relationship is to a high degree complicated by the development of tolerance. The concentration-response curve might differ between acute and chronic administration of nitrates. Tolerance might develop toward some of the effects but not to others.

6. The pharmacodynamics of the nitrates might be more dependent on the rate and direction of changes in plasma concentrations of the drugs rather than on the absolute values of the plasma concentration.

7. Initial effects may be attenuated by counterregulatory mechanisms, e.g., activation of adrenergic activity, the renin-angiotensin system, or other mechanisms.

VI. In Vivo Evaluation of Effects of Nitrate Esters on Different Target Organs

Despite the fact that nitrates have been used as a remedy for angina pectoris for more than a century, the exact mechanism(s) of their antianginal effect is not clear. Several mechanisms acting via different target organs (vessels, heart, platelets) contribute to the effect. The relative importance of these mechanisms (particularly in a single patient) is not known, and it can be difficult to specify the predominant mechanism. However, it is well documented that the relaxing effects exhibited by the nitrates on vascular smooth muscle in veins, coronary arteries, and peripheral resistance vessels are of importance.

A. Peripheral Hemodynamic Effects

The basic hemodynamic effect of the nitrates is vasodilation of both venous and arterial vessels. It has been shown in experimental animals (Bassenge et al., 1981), in normal human volunteers (Imhof et al., 1980), and in patients with coronary artery disease (Miller et al., 1976; Gerson et al., 1982) that the venous capacitance vessels have the highest sensitivity toward organic nitrates and, hence, there is a dominating preload reduction at low doses/concentrations. Splanchnic veins, together with veins of the arms and legs, are particularly important for the venous pooling effect of nitrates (Loos et al., 1983; Strohm et al., 1983).

Venous vessels might have a higher sensitivity toward organic nitrates than arterial vessels (Mackenzie and Parratt, 1977; Nakajima and Nosaka, 1983; Toyoda et al., 1986; Rösen et al., 1987). It has been shown in vitro that ISDN, IS-5-MN, and GTN inhibited Ca release from Ca stores more effectively in the rabbit femoral vein than in the rabbit femoral artery (Toyoda et al., 1987). In addition, the difference might be a consequence of the concentrations of nitrates in the tissues of the venous capacitance vessels being higher than those on the arterial side. Fung et al. (1984) observed that the uptake of GTN, when given as a bolus injection to rats, seemed to be higher in the vena cava than in the aorta.

The venodilating effect of nitrates leads to a reduction of the left ventricular end-diastolic pressure and end-diastolic volume and, therefore, unloading of the heart and lowering of the oxygen consumption. In patients with coronary artery disease, a reduction in left ventricular end-diastolic volume of between 10 and 40% has been observed (Battock et al., 1976; Salel et al., 1976; Ritchie et al., 1979; Pfisterer et al., 1983; Kaski et al., 1985). During exercise, a mean left ventricular end-diastolic volume reduction of about 20% was reported after patients with coronary artery disease received GTN sublingually (Pfisterer et al., 1983). In resting patients with coronary artery disease, the volume changes are reflected by a mean increase in left ventricular ejection fraction from 43 to 63% (Steele et al., 1978). During exercise, sublingual GTN increased left ventricular ejection fraction from 50 to 60% (Pfisterer et al., 1983) and from 36 to 48% (Borer et al., 1978).

In dose-response studies of nitrates, it has been found that higher doses are required to change arterial tone as compared with venous tone (Gwilt et al., 1983). The arteriolar resistance vessels are dilated only at high concentrations of organic nitrates (Bassenge et al. 1981); in the usual therapeutic range, however, the systemic arterial resistance remains largely unaffected (Strauer and Scherpe, 1978; Wille et al., 1980; Kober et al., 1981).

The documented nitrate-induced decrease of the afterload leads to an unloading of the heart and eventually to reduced oxygen consumption. The effect must be due to either an increased aortic compliance (Martin et al., 1976) or an increase of the Windkessel function, without alteration of aortic distensibility (Wille et al., 1980). The influence on the Windkessel function may be mediated not only by the aorta itself but also by large muscular type branches such as the mesenteric and hepatic arteries (Strohm et al., 1983).

Effects of higher doses of nitrates differ between patients with an increased peripheral resistance and those

with normal peripheral resistance; increases in cardiac output are seen in patients with severe congestive heart failure (Packer, 1985). The presence of edema seems to decrease the preload-reducing effects of nitrates (Magrini and Niarchos, 1980, 1983). Moreover, all nitrates may not have the same effects on different vascular systems. Rezakovic et al. (1983) proposed that GTN is a more potent venous dilator than is ISDN, which they considered a mixed vasodilator. In contrast, there are in vitro studies that have shown that ISDN and IS-5-MN are more venoselective than GTN (Stiefel and Kreye, 1984; Toyoda et al., 1986).

In studies of animals (Bassenge et al., 1981) and patients (Schneider et al., 1986), no differences were found between GTN and IS-5-MN and GTN and ISDN, respectively, regarding their venoselectivity.

Roth et al. (1987) evaluated the effects of intravenously administered ISDN and GTN on hemodynamics in patients with acute myocardial infarction and elevated pulmonary artery wedge pressure. They showed that doses of ISDN and GTN producing a comparable reduction in pulmonary artery wedge pressure also had similar hemodynamic effects, including effects on systemic vascular resistance.

Regarding clinical studies in general, there is no firm evidence available that selectivity for different vascular beds varies for the different organic nitrate esters. It must be stressed, however, that this has not been extensively studied. One smaller-scale study has been carried out in patients with chronic congestive heart failure receiving infusions of IS-5-MN and GTN. Both drugs were individually titrated to achieve a maximal increase in cardiac output and a maximal decrease in capillary wedge pressure. IS-5-MN produced a greater decrease in PCWP and a smaller decrease in systemic arterial pressure (Schneeweiss, 1988). This might indicate that a difference such as that discussed above may exist, a finding that may be of clinical importance.

If such a dissimilarity in venoselectivity really exists between the different nitrate esters, it might be of clinical interest. Patients expected to benefit mainly from venodilation should perhaps be treated with IS-5-MN or ISDN rather than GTN. A difference in venoselectivity might also be of significance for the induction of counterregulatory mechanisms with a possible impact on attenuation of the hemodynamic effect (and the therapeutic effect) in long-term treatment (Stewart et al., 1986, 1987; Packer et al., 1986; Bennett and Davis, 1985).

It also must be remembered that there are huge interindividual variations in the vasodilating effects of nitrates, particularly GTN. Eichler et al. (1987) studied the effect of a local infusion of GTN using a dorsal hand vein compliance technique. The GTN concentration that caused a 50% venodilation ranged from 0.2 to 22.4 ng/min, representing a 100-fold variation in the response of the effector organ. In the investigation, two different age groups participated, one with a mean age of 23 years and the other 63 years, but no differences were apparent between the two groups. Gascho et al. (1989) used a plethysmographic method to measure venous distensibility and found a difference between younger and older subjects. This does not necessarily mean that the dosage of GTN has to be increased in older subjects; in fact, there are some indications that GTN might be a more efficacious agent in elderly versus younger patients, a finding that might be related to a lower circulating plasma volume (Marchionni et al., 1988). However, it should be kept in mind that the patients in the study by Marchionni et al. (1988) suffered from heart failure and recent transmural myocardial infarction, and it might be impossible to extrapolate the data to other healthier subjects.

Except for the direct vascular effects, it has been proposed that nitrates influence left ventricular diastolic properties. This beneficial effect is, however, probably due to the vasodilating properties of the nitrates (Ludbrook et al., 1977; Amende et al., 1983; Kingma et al., 1986). Hirzel et al. (1983) reported that IS-5-MN improved left ventricular relaxation, either directly or by relief of myocardial ischemia; the effect was achieved without any change in myocardial contractility.

Direct effects of nitrates on left ventricular contractility have also been proposed, but these are most likely due to the anti-ischemic effect (Harris et al., 1983). McAnulty et al. (1975) reported that sublingual administration of GTN diminished abnormalities in left ventricular wall motion in patients with coronary artery disease. Several studies have confirmed that nitrates improve the function of viable hypokinetic myocardial segments but not that of nonviable dyskinetic myocardial segments (Reddy et al., 1975; Dumesnil et al., 1975; Bodenheimer et al., 1976). Regional left ventricular contractile responses to sublingually administered GTN have been shown to accurately predict improvement in regional function after coronary bypass surgery which restores the blood supply to these segments (Helfant et al., 1974; Chesebro et al., 1976).

In a study of patients with left ventricular dyskinetic segments in the absence of symptoms of myocardial ischemia (silent ischemia), Pepine and coworkers (1986) demonstrated that the presence of dyskinesia was associated with the presence of significant coronary artery disease in a vessel supplying that segment. GTN improved contractility in these segments, which might be taken as an indication that silent ischemia can be reversed by GTN (see VII.C.2).

In conclusion, organic nitrate esters relax vascular smooth muscle and dilate peripheral vessels on both the venous and arterial sides. Venous capacitance vessels are most sensitive, and there is a dilation at low doses leading to an unloading of the heart and a decrease of the oxygen consumption in the myocardium. The dilation of arterial

resistance vessels occurs at higher concentrations and results in an unloading of the heart by a decrease in afterload.

B. Effects on Coronary Vessels

The vasodilating activity of GTN in isolated coronary arteries was first described in 1913/1914 by Voegtlin and Macht. Since that time, the importance of coronary vasodilation for the antianginal activity of nitrates has been a matter of dispute. In animal experiments, a biphasic response to nitrates in coronary flow was observed; a short-lived pronounced increase of flow was followed by a longer-lasting decrease of flow as a consequence of an increase in total coronary resistance (Rees et al., 1966; Winbury et al., 1969). Similar observations have been made in patients (Cowan et al., 1969).

The finding of Ganz and Marcus (1973) that an intracoronary injection of GTN was unable to alleviate pacing-induced angina, whereas i.v. administration promptly relieved it, in patients with coronary artery disease, was taken as evidence against the importance of coronary dilation for the therapeutic effect of nitrates. However, the intracoronary bolus dose used was 0.075 mg, which is very high and, therefore, the potential beneficial effect of GTN resulting from a selective dilation of the large coronary arteries was probably counteracted by effects of the autoregulatory processes. Feldman et al. (1982) have shown that coronary artery dilation, without changes in heart rate or systemic hemodynamics, occurs at a 5-μg dose of GTN intracoronary.

There are at present a number of reports showing that nitrates have a direct effect on coronary artery dilation, which leads to an increase in the oxygen supply to the myocardium. Intracoronary doses of GTN have been found to increase the diameter of normal left anterior descending and left coronary artery by 9 to 22% and that of collaterals by 18 to 28% (Feldman et al., 1979). Other studies have confirmed this. For example, Brown and coworkers (1981) found an average increase of 18% in cross-sectional area of normal coronary segments after sublingual administration of GTN. The increase was greater (35%) in large vessels (1.6 to 2.3 mm in diameter) but only 9% in small vessels (0.4 to 0.5 mm in diameter). They also demonstrated that GTN dilated stenotic lesions in large coronary arteries. The preferential effect of GTN on large coronary arteries has been confirmed by other investigators (Schnaar and Sparks, 1972; Harder et al., 1979).

It has recently been demonstrated that a low dose of GTN given intravenously is able to dilate coronary artery stenoses. In a randomised double-blind study, 40 patients with coronary heart disease received either placebo or 0.025 mg GTN i.v. Before and 2 to 3 min after injection, the aortic and left ventricular pressures were recorded, and coronary angiography was performed. No significant changes were observed in mean heart rate, systolic and diastolic aortic pressure, left ventricular filling pressure, and the pre- and poststenotic coronary artery diameters. Coronary artery stenosis diameters remained unchanged after placebo but increased significantly after GTN (Sievert et al., 1989). The authors concluded that dilation of coronary stenoses plays an important role in the antianginal action of GTN.

Rafflenbeul et al. (1980) showed that 5 mg ISDN given sublingually dilates normal epicardial arteries and that large stenosed arteries may be subject to a substantial increase in diameter. Badger et al. (1985) studied the response to sublingually administered ISDN (5 or 10 mg) in 10 men with suspected coronary artery disease as they were undergoing coronary arteriography. Quantitative arteriography demonstrated substantial dilation of luminal cross-sectional area in both normal and diseased coronary arterial segments. Diseased epicardial segments (stenosis ≥50%) dilated 51% ($P < 0.01$) after 10 min, and calculated stenosis resistance decreased 40% ($P < 0.01$). Diseased segments in small- and middle-sized arteries (1 to 8 mm^2) were four times more reactive than those in larger arteries (>8 mm^2); these responses occurred within 3 min after ISDN administration, peaked at 10 min, and persisted, unchanged, for the remaining 20 min. Using quantitative magnification coronary angiography in patients treated with GTN (0.4 mg sublingually), Feldman et al. (1981) found that the coronary vasodilation was inversely related to the diameter of the arteries.

There is also an investigation indicating that organic nitrate esters do more than dilate the coronary arterial bed. GTN, 0.2 mg administered directly into the left coronary artery, produced a transient decrease in coronary resistance and a subsequent increase in coronary flow, as expected, but, in addition, there was a marked increase in coronary vascular volume, which was still present when the effect on the coronary arteriolar bed had subsided, indicating a more widespread dilatory effect of GTN on the coronary circulation (Simon et al., 1987).

De Coster et al. (1990) compared the effects of ISDN administered by intracoronary and i.v. routes in 10 patients with severe coronary artery disease, stable effort angina, and low exercise tolerance. After intracoronary infusion of ISDN, ST-segment depression and the increase in left ventricular end-diastolic pressure and left ventricular end-systolic volume induced by exercise were significantly less abnormal than during control conditions. When exercise was performed after i.v. infusion of ISDN, the above-mentioned parameters were significantly improved even further. The authors conclude that exercise-induced ischemia is relieved mainly by the peripheral effects of nitrates but that there is also a direct effect on coronary blood supply.

In conclusion, today, it is accepted that the direct effects on coronary circulation exerted by the nitrates contribute to the antianginal effects of the drugs, not

only in vasospastic angina (Engel and Lichtlen, 1981; Hillis and Braunwald, 1978) but also in exertional angina, especially because it now is evident that active vasoconstriction of stenotic segments in patients with exertional angina takes place during dynamic exercise (Krayenbühl et al., 1987).

Experimental results also indicate that organic nitrate esters might have a direct effect on myocardial metabolism (Gross and Hardman, 1975; Szekeres et al., 1978). A number of studies of isolated rat atria have shown a beneficial effect of GTN on myocardial metabolism. Under experimental conditions mimicking ischemia, GTN tended to normalise the metabolism (Laustiola et al., 1983b), and the same effect has been suggested to occur in vivo (Kedem et al., 1985a,b) and in patients (Bagger et al., 1984). A study by Borow and coworkers (1981) showed a reduction of metabolism which was independent of mechanical performance; these authors used a myothermal technique that permits direct estimation of myocardial energetics.

To characterise regional myocardial perfusion and glucose metabolism in unstable angina and the effect of ISDN on these parameters, Araujo et al. (1987) studied 22 patients with angiographically proven coronary artery disease and severe unstable angina by using positron emission tomography. Myocardial glucose utilisation was found to be increased in these patients, even in the absence of clinical signs of acute ischemia or detectable perfusion abnormalities. Intravenous administration of ISDN reduced myocardial oxygen utilisation, suggesting a beneficial effect on myocardial metabolism.

Determining to what extent nitrates exert a direct effect on myocardial metabolism deserves further study.

C. Effects on Platelets

A number of studies have shown an in vitro inhibition of platelet aggregation by GTN (Schaffer et al., 1980; Ahlner et al., 1985; Loscalzo, 1985). However, this has only been seen when using concentrations in the millimolar range (i.e., at least 100,000-fold greater than what is considered to be the therapeutic plasma concentration), unless exogenous SH groups were added (Loscalzo, 1985). In an experimental study of pigs, Lam et al. (1988) investigated the effect of GTN on platelet disposition in common carotid arteries injured by balloon angioplasty. It was found that, in the presence of deep arterial wall injury, a dose of GTN sufficient to lower mean arterial blood pressure by 9% significantly decreased platelet disposition relative to control.

Earlier in vivo studies failed to demonstrate that GTN has an effect on platelet aggregation (Fitzgerald et al., 1984) or on experimentally induced coronary artery thrombosis in dogs (Martorana et al., 1984). However, there are reports that have indicated a possible effect of therapeutic doses/concentrations on blood rheology (Brügger et al., 1985) and on bleeding time (Lichtenthal et al., 1985).

There are few reports published regarding the beneficial effect of therapeutic doses of GTN on platelet function in humans (Diodati et al., 1988, 1990; Karlberg et al., 1990). However, other researchers have been unable to show such an effect, even in combination with N-acetylcysteine (Hogan et al., 1989a). Hogan et al. investigated the effect of GTN, given with and without N-acetylcysteine, on ex vivo platelet aggregation in eight healthy volunteers. In a double-blind randomised crossover trial, the subjects were treated transdermally with 20 mg GTN/24 h, together with N-acetylcysteine 200 mg three times daily or matching placebo. Platelet aggregation, measured ex vivo by whole blood impedance aggregometry in response to ADP, was not significantly altered by GTN, neither acutely nor after 4 days of treatment with or without N-acetylcysteine. Platelet guanosine monophosphate levels were not significantly altered by GTN, either in the absence or in the presence of N-acetylcysteine.

Stamler et al. (1988) investigated the effect that GTN, given intravenously to patients, had on platelets studied ex vivo. They found that in vivo infusions of GTN titrated to hemodynamic response or clinical effect did not significantly affect ADP-induced platelet aggregation ex vivo. However, increasing concentrations of N-acetylcysteine added ex vivo progressively inhibited ADP platelet aggregation in all patients tested, both before and after GTN infusion. The authors concluded that platelets taken from patients treated with i.v. GTN manifest attenuated aggregation responses ex vivo when thiol stores are repleted and that N-acetylcysteine per se inhibits platelet aggregation in a dose-dependent fashion.

Diodati et al. (1990) studied the platelet aggregation response to ADP and to thrombin before, during, and after a 45-min infusion of GTN in 10 patients. An impedance aggregometer allowing rapid beside studies in whole blood was used. When the aggregation was tested immediately, there was >50% inhibition in all patients. When analyses were delayed and performed on blood preserved at room temperature for 30 min, no effect of GTN could be detected.

A clinical investigation of ISDN showed a marked decrease in platelet aggregation when the drug was infused at a rate of 4 mg/h to patients with angina pectoris (De Caterina et al., 1984). In this study it was found that, in vitro, ISDN was not able to inhibit platelet aggregation at the low concentrations sufficient for inhibition in vivo.

In another investigation De Caterina and coworkers (1988) showed that the ISDN metabolite IS-2-MN was somewhat more active in vitro than ISDN in inhibiting platelet aggregation. The clinical effect might be due to this metabolite. The activity of the other metabolite, IS-5-MN, was much less, and it did not show any inhibitory effect on platelet aggregation in concentrations of <0.1 mM.

However, in a recent in vivo study of nine patients

with stable coronary artery disease, De Caterina et al. (1990) showed that IS-5-MN, as well as IS-2-MN, might inhibit platelet aggregation. The patients were given an infusion of both IS-2-MN and IS-5-MN for 30 min on two separate days, at rates of 4 and 8 mg/h, respectively. A significant decrease in platelet aggregation and thromboxane B_2 production by ADP and adrenaline occurred in seven of nine patients receiving IS-2-MN and in 7 of 11 patients receiving IS-5-MN. Responses were dose related with more patients responding to IS-2-MN at 8 mg/h (five of five) than to IS-5-MN (three of five) and with a maximum at the end of the infusion time corresponding to peak plasma levels. Patients responding to drug infusions with an inhibition of platelet function were characterised by a greater vascular responsiveness compared to nonresponders, because the decrease in systolic blood pressure was significantly greater in the former (15.4 mm Hg) than in the latter (2.5 mm Hg).

In conclusion, vasodilation caused by nitrates is of utmost importance for the antianginal and anti-ischemic effects seen in different vascular beds, in capacitance vessels on the venous side, and in resistant vessels on the arterial side. Both of these mechanisms eventually lead to a decreased oxygen consumption in the myocardium. The dilation of the coronary vessels increases the oxygen supply to the myocardium. There is patient to patient variation with regard to which of the vascular beds the nitrates are exerting their most beneficial action. The possible effects of nitrates on platelet aggregation and myocardial metabolism have not yet been sufficiently studied to allow evaluation of their importance in clinical practice.

VII. Clinical Therapeutic Use of Organic Nitrates

A. Stable Angina Pectoris

Sublingually administered GTN is still the cornerstone in the treatment of an attack of angina pectoris. A growing number of different formulations of GTN have been developed for prophylactic use. ISDN has long been used for the same purpose, and during the last few years IS-5-MN has become a third alternative in the prophylaxis of angina.

1. Glyceryl trinitrate. The value of sublingual administration of GTN in the treatment of angina pectoris attacks is undisputed. GTN can be administered in the form of sublingual tablets or oral spray; the onset of action is rapid, within 1 to 3 min; the duration is limited to 20 to 30 min.

It has been shown that an increase in exercise tolerance in patients with angina pectoris is dose dependent, irrespective of whether tablets or oral spray are used (Parker et al., 1986). Based on this it was concluded that the spray formulation of GTN was equivalent to sublingual tablets in aborting episodes of angina. In another dose-response study, sublingual tablets of 0.25 to 0.75 mg GTN were given to patients before bicycle exercise testing (Nyberg and Holmberg, 1986). The lowest dose (0.25 mg) had no significant antianginal effect compared to placebo, measured as time until onset of chest pain or ST-segment depression during exercise; in contrast, 0.5 and 0.75 mg were effective. The highest dose produced a greater decrease in blood pressure but did not show any improved antianginal effect; thus, 0.5 mg was considered the optimal dose. It should, however, be kept in mind that some patients may be more sensitive to GTN (hypotension, headache) and will tolerate only lower doses.

A buccal formulation of GTN has been developed that combines a rapid onset with a prolonged effect (up to 4 to 5 h), provided the patient keeps the tablet in the mouth. It is intended for so-called situational prophylaxis but can also be used to abort episodes of angina. In a Swedish multicentre study, buccal and sublingual GTN were compared in 126 patients (Rydén, 1987). The study concluded that the two formulations are comparable in the treatment of acute attacks of angina pectoris. However, the cost per dose for the buccal formulation is considerably higher than that for sublingual tablets or spray.

The described buccal formulation of GTN has been shown to be effective in preventing anginal attacks. The effect was persistent after 2 weeks of treatment when given three times daily as a 3-mg dose (Parker et al., 1985). The effective duration after a single dose is usually 4 to 5 h but was, in one study, as many as 7 h (Grasso et al., 1988).

The effectiveness of GTN administered by mouth as sustained-release formulations has been documented in a number of studies (Winsor and Berger, 1975; Davidov and Mroczek, 1977; Degré et al., 1983; Berkenboom et al., 1984; Schneider et al., 1984).

Winsor and Berger (1975) performed a double-blind, randomised, crossover trial with sustained-release GTN (2.6 mg, given three times daily) against placebo, during 6 months and with randomly sequenced 1-month treatment periods. GTN reduced the incidence and the severity of anginal attacks by about 50% and reduced the consumption of sublingual GTN. In 15 patients, ST-segments were continuously monitored for 10 to 12 h during long-term treatment under normal life-style conditions while receiving placebo and GTN. There was significantly less ST-segment depression in patients treated with GTN than with placebo.

Winsor and Berger also tested, the effect of GTN on exercise tolerance in 22 other patients. The exercise tests were performed at least 4 h after the GTN dose. GTN significantly prolonged the time to onset of angina and significantly reduced the duration of angina. In this study the question of tolerance was addressed by counting the consumption of sublingual GTN every week during an 8-week treatment period. The consumption of GTN was not greater at week 8 when compared with week 1.

In an another double-blind, randomised study, oral

sustained-release GTN, in doses of 19.5 to 39 mg/day, led to a significant decrease in the frequency of anginal attacks and a considerable increase in exercise capacity (Davidov and Mroczek, 1977).

Degré et al. (1983) compared 2.5 and 6.5 mg GTN with placebo in a double-blind crossover fashion. The effect was evaluated by bicycle exercise 1 and 5 h after a dose. The dose of 2.5 mg was effective on the symptom-limited working capacity only at 1 h, whereas 6.5 mg was effective at 5 h as well.

The dose-response relationship for anti-ischemic activity and dose was studied by Schneider et al. (1984). They treated 12 patients with doses of 2.6, 6.5, 10, and 20 mg, and the effect was evaluated by exercise ECG. The ischemic reaction (sum of ST-segment depressions) was not significant for the smallest dose (2.6 mg) compared to placebo but was significant for the other doses at 30 min after the treatment. The effect was directly dose related and was still demonstrable 3 h later. The highest dose had a significant effect compared to placebo, even 9 h posttreatment.

Berkenboom et al. (1984) studied the effect of sustained-release GTN, 6.5 mg, given three times daily to 46 patients in a randomised, placebo-controlled, double-blind trial lasting 2 weeks. They found a slight, but statistically significant, decrease in the number of anginal episodes and in the consumption of sublingual GTN. An exercise test 1 h posttreatment revealed that exercise duration increased and that symptom-limited exercise and ST-segment depression was significantly decreased during GTN therapy as compared to placebo.

In conclusion, some studies do show an acute effect and even long-term effects of oral GTN in sustained-release form, although these investigations were not properly designed to address the question of development of tolerance. The effective doses seem to differ greatly among individual patients; there is probably not enough effort made in clinical practice to find the right dosage for a specific patient, resulting in many patients being treated with too low a dose.

Cutaneous exposure to GTN has long been known to produce pharmacodynamic effects (Crandall et al., 1931). In the 1940s, a GTN ointment was introduced as a remedy for peripheral circulatory disturbances (Lund, 1948), and in the 1950s, GTN was reported to be an effective antianginal agent (Davis and Wiesel, 1955). Davidov and Mroczek (1976) studied the effect of a 2% ointment and placebo on exercise tolerance in 12 patients with coronary disease. Significant antianginal efficacy could be seen for at least 3 h. In a randomised, double-blind study of 10 patients with angina pectoris, Karsh et al. (1978) found a significantly increased exercise capacity as well as a reduction in ST-segment depression throughout at least 3 h after administration of 5 to 8 cm of a 2% GTN ointment. Davidov (1981) reported that an antianginal activity of as many as 8 h might be achieved by applying 2 cm of a 2% GTN ointment over an area of 6 × 8 cm.

In a double-blind crossover study against placebo, Kala et al. (1983) showed that application of an ointment containing 15 mg GTN improved the total work performed in an exercise test for ≤7 h after application. There was also a study showing a prolonged effect during long-term drug treatment (Reichek et al., 1974).

The clinical usefulness of the ointment formulation is limited by the fact that it can be difficult for the patient to apply the right dose, and many patients find the ointment inconvenient to handle.

The development of transdermal GTN discs aimed at 24-h prevention of anginal episodes created the first practical basis for keeping constant plasma levels of the drug in ambulatory patients. Studies of the efficacy of GTN patches have yielded conflicting results regarding which dose is necessary to obtain a given effect and concerning the duration of that effect. Bennett and Davis (1985) performed a hemodynamic study of healthy volunteers and proposed that the commonly used doses of GTN in the patches were too small to produce significant antianginal effects. However, most studies have shown an initial effect, even with small doses, the problem instead being a rapid attenuation of the initial effect.

There are, indeed, a number of studies that have failed to demonstrate a preservation of the effect during 24 h. As early as the year following the "conditional approval" of the GTN patches by the Food and Drug Administration, there were reports indicating that the effect did not persist for 24 h (Reichek et al., 1983; Crean et al., 1983).

The hemodynamic and antianginal efficacy of transdermal GTN patches in patients with chronic stable angina was studied by Parker and Fung (1984). They found that patches designed to deliver 5, 10, and 15 mg of GTN/24 h improved treadmill walking time 2 and 4 h after application, but no effect was seen at 24 h. These results were confirmed by other investigators (Reichek et al., 1984; Thadani et al., 1986; Sullivan et al., 1985), and the explanation suggested was a rapid (within 24 h) development of tolerance. In a randomised controlled trial in 427 men with chronic stable angina, Fletcher et al. (1988) studied the efficacy (anginal attack rates and sublingual GTN consumption) and the effect on quality of life (measured with the sickness impact profile and a health index of disability) of the continuous use of 5 mg transdermal GTN as compared to placebo. Active treatment showed no advantage over placebo, and patients receiving the active drug reported headaches more frequently than patients receiving placebo.

The Food and Drug Administration-initiated multicentre trial of transdermal GTN in angina pectoris, using doses from 15 to 105 mg/24 h, versus placebo, is now completed, but the results have not yet (as of September 1990) been published. The results from this study comprising >500 subjects show that, with long-term or daily

use, all treatment groups lost the beneficial first dose antianginal effect, which, moreover, occurred only at the 4-h postdose exercise test on the first day of therapy (Abrams, 1989).

A meta-analysis of randomised clinical trials of transdermal GTN systems by Colditz at al. (1988) came to the same conclusion; this analysis combined the results of 17 trials. At 3 to 4 h after patch application, there was a significant increase in exercise duration for both a dose of 5 mg/24 h (77 s; $P = 0.02$) and 10 mg/24 h (114 s; $P = 0.001$). At 24 h, however, the increase in exercise duration was not significant. Other studies have shown that the GTN plasma level is maintained for 24 h (Muller et al., 1982; Colfer et al., 1982; Imhof et al., 1984).

There are investigators who have reported improvement in exercise tolerance for as many as 16 or 24 h (Schiavoni et al., 1982; Pucci et al., 1983; Cerri et al., 1984). Those studies used open-label or single-blind techniques. There are, however, a few double-blind studies in which improvement in exercise tolerance for as many as 24 h were observed (Naafs et al., 1984; Scardi et al., 1985).

The discrepancy among different studies might be explained by differences in the selection of patients, the number of patients, etc. There seem to be huge interindividual differences in the response to treatment with GTN patches as shown by Muiesan et al. (1986) in a double-blind, randomised, placebo-controlled crossover comparison between a patch delivering 10 mg GTN in 24 h and a placebo patch in 57 patients with stable effort angina. Active treatment significantly increased exercise tolerance for the whole group; however, when the results obtained in individual patients were analysed, only about 50% of the patients responded to treatment.

Intermittent therapy has been tested as a means of obtaining a persistent antianginal effect during long-term transdermal administration of GTN. Cowan et al. (1987a,b) found that 12-h daily intermittent therapy resulted in a sustained effect after 1 week, whereas continuous (24-h/day) treatment gave an attenuated effect after the same duration. However, in the two studies the evaluation using an exercise test was carried out 3.5 h after application only.

In a study by Schaer et al. (1988), exercise end points were significantly improved in 11 patients tested 4 and 8 h after patch application compared with 10 h/day without a patch. Luke et al. (1987) showed that intermittent, but not continuous, therapy remained effective; patients were tested 2 and 4 h after patch application.

In a randomised double-blind evaluation of 206 patients in whom treadmill exercise was used, two different transdermal GTN doses, 5–10 and 15–20 mg/24 h, were tested against placebo (DeMots et al., 1989). Treadmill testing was performed 0, 4, 8, and 12 h after patch application, at baseline (day 0) and on days 1, 15, and 29. After short-term application both doses were significantly better than placebo 12 h postapplication. The improvement in the treadmill walking time was largely lost after 4 weeks with the lower doses.

In a randomised, placebo-controlled trial with 36 patients with stable angina, continuous and intermittent (18 or 14 h/day) treatment with transdermal GTN, 10 mg/day, was compared. Exercise tests were performed during the last 2 h of patch application. Compared with placebo, neither continuous GTN nor the two intermittent regimens prolonged total treadmill time or time to 1-mm ST-segment depression (Waters et al., 1989).

The possibility of using intermittent administration has lead to the introduction of phasic-release GTN systems designed to produce biphasic plasma GTN concentrations. The release profile provides maximal plasma GTN at approximately 4 h, with therapeutic plasma levels during the initial 12-h period and lower concentrations during the remaining 12 h (Parker, 1989).

In conclusion, transdermal patches continuously releasing GTN throughout 24 h induce tolerance rapidly in many patients. The use of intermittent therapy seems to reduce the risk of tolerance development, but the results are somewhat conflicting. Further investigations are warranted to establish the effectiveness of intermittent therapy and the risk for rebound phenomena (see VIII).

2. Isosorbide dinitrate. Several studies have demonstrated that sublingually administered ISDN has a longer duration of action than does GTN. Kattus et al. (1979) investigated the protective action of 5 mg ISDN compared with 0.4 mg GTN administrated sublingually on treadmill-induced angina: the effect of ISDN persisted for 2.5 to 3 h but <1 h for GTN. In a study by Steele et al. (1978), the effects of 5 mg sublingual ISDN on the ST-segment depression during exercise was found to be sustained for 3 h in 15 patients with coronary artery disease. In an early study by Goldstein et al. (1971), the duration of effect of sublingual ISDN was not longer than for GTN administered sublingually when the effect was tested 10 min and 1 and 2 h after dosing. However, as shown by the other studies and according to clinical experience, the duration of effect of sublingual administered ISDN is longer than that of GTN.

ISDN is also available as an oral spray which was shown to have a more rapid onset of effect and is, therefore, better suited for relief of anginal pain (Reisin et al., 1988).

Glancy et al. (1977), in a double-blind crossover experiment, studied the effects of both a small (5 to 10 mg) and a large oral dose of ISDN (10 to 30 mg) and placebo on patients with coronary artery disease. The hemodynamic effects became apparent after 15 min and were present until 3 h after the small dose and were still significant 4 h after the larger dose. Exercise capacity was clearly increased 2 h after oral administration of 7.5 to 20 mg ISDN. Lee et al. (1978) showed a persistent

antianginal effect of ISDN given orally in a dose of 40 mg every 8 h.

Markis et al. (1979) showed, in a randomised, double-blind crossover study against placebo, that 20 mg of ISDN increased exercise tolerance and reduced ST-segment depression for at least 3 h. Danahy et al. (1977) showed that acute oral administration of a mean dose of 29 mg ISDN to 21 patients with angina pectoris increased the net exercise time during a period of as many as 5 h; the study was double blind, randomised, and crossover in design. The patients in this study were retested after a mean period of 5.6 months, and the increase in exercise time up to 5 h persisted, although the hemodynamic changes were attenuated.

Parker et al. (1987a) showed that 30 mg of oral ISDN increased treadmill walking time during a 5-h observation period and that this effect was maintained when the drug was administered twice or thrice daily but not when given four times daily with the last dose given at 11 p.m.

Recently, a study by Bassan (1990) indicated that during long-term treatment (2 weeks or longer) the duration of the effect on exercise capacity of a titrated dose of ISDN (27.5 mg) given orally to patients with stable angina pectoris was not >3 h after the administration of the dose. There seemed to be a progressive attenuation of the antianginal effect throughout the day. The third dose given at 6 p.m. had a very modest effect even 1 h posttreatment. The study was double blind and placebo controlled, but only 8 patients took part and the study needs confirmation.

ISDN ointment (100 mg) has been shown to be effective for as many as 8 h after acute administration, whereas the effect does not persist in long-term treatment (Parker et al.,.1984).

3. Isosorbide-5-mononitrate. IS-5-MN is the principal active metabolite of ISDN. It has been marketed as a drug in its own right mainly because of the pharmacokinetic properties that make it suitable for oral administration, thereby providing prophylaxis against angina pectoris.

The antianginal effect of IS-5-MN has been documented in both acute and long-term studies. In a double-blind, randomised, placebo-controlled trial, Reifart et al. (1981) were able to show that acutely administered IS-5-MN (20 mg) is approximately as active as 20 mg of sustained-release ISDN in reducing the exercise-induced ST-segment depression in patients with stable angina.

In a double-blind, randomised, three-way crossover study against ISDN and placebo, Muller et al. (1983a) demonstrated the antianginal activity of IS-5-MN during chronic treatment in 18 coronary patients. In another double-blind, randomised,crossover study by Muller et al. (1983b), it was shown that the improvement in exercise capacity and reduction of ST-segment depression persisted for up to 10.5 h after 20 mg of oral IS-5-MN.

In a double-blind, randomised, crossover study by Thadani et al. (1987c), the duration of effects of single oral doses of 20 and 40 mg IS-5-MN and matching placebo were studied in 12 patients with angina pectoris. Exercise tests were performed at 2 and 6 h, and both doses produced a significant increase in exercise time to the onset of angina and in total exercise duration at 2 and 6 h; no significant difference was seen between the two doses tested. Recently, De Belder et al. (1990) showed that the antianginal and anti-ischemic effects of IS-5-MN (20 mg administered orally) persisted for as many as 8 h but not up to 10 h.

The optimal single dose of IS-5-MN seems to be 20 mg for most patients. Abshagen and Spörl-Radun (1981) showed that 20 mg gave an optimal vasodilatory effect as studied by finger plethysmography. The same observation applies to the hemodynamic effects in normal volunteers as well as in patients with angina (Jansen et al., 1985). Regarding the antianginal activity, there are other studies, in addition to the one by Thadani et al. (1987c), that have indicated that the optimal single dose of IS-5-MN is 20 mg (Akhras et al., 1985; Jones et al., 1987; Jansen et al., 1988).

A number of investigations have demonstrated that IS-5-MN given twice daily in a 20-mg dose has a persistent antianginal activity during long-term treatment (Akhras et al., 1985; Rennhak et al., 1985; Mefert and Paeckelmann, 1987; Uberbacher et al., 1983). Sustained-release formulations of IS-5-MN, intended to be administered only once a day, are available. Nyberg et al. (1986) showed in a double-blind crossover study against placebo that IS-5-MN (60 mg in a controlled-release formulation given once daily) significantly increased exercise tolerance until the onset of angina and until the appearance of 1-mm ST-segment depression; this occurred both after the first dose and after 1 week of treatment. In a recent study by Wisenberg et al. (1989) 60 mg sustained-release IS-5-MN given once daily showed a sustained antianginal effect during 11 to 14 days of treatment.

In conclusion, to abort anginal attacks, sublingually administered GTN is still the drug of choice. Buccal administration of GTN, intended to provide situational prophylaxis, might be suitable for more patients than are actually treated with this formulation today. For prophylactic use, orally administered IS-5-MN or ISDN seem to be the best choices; dose titration studies have been more properly performed for these substances than for oral GTN, and tolerance development has been studied. Whether slow-release preparations possess any major advantages remains to be settled. In certain patients transdermal GTN might be a suitable, although expensive, alternative. The topic of dosage regimens minimising the risk for tolerance development is discussed in section VIII.

B. Vasospastic Angina

The term "coronary spasm" is often used, although it has no uniform definition. However, two concomitant

occurrences are required for the diagnosis of coronary artery spasm: reversible and objective evidence of myocardial ischemia and reversible coronary vasoconstriction (Conti and Curry, 1980). Coronary vasospasm in patients with normal or near normal coronary arteries is a rare form of angina (Heupler, 1980). Mixed forms of angina are more common and are most often seen in patients with severe coronary heart disease; a vasospastic component has been estimated to be of importance in 14 to 20% of patients with this form of angina (Bertrand et al., 1982).

1. Glyceryl trinitrate. GTN ointment (60 mg/day) was evaluated in a controlled short-term study of 10 patients with angina at rest and ST-segment elevations (Salerno et al., 1981). The anginal attack rate was reduced by 85% in the GTN-treated group. Many patients with coronary spasm respond completely to a conventional sublingual dose of GTN (Nelson et al., 1977; Baumann, 1978). In patients for whom sublingual GTN is less effective, i.v. GTN is of value (Schroeder et al., 1977; Epstein et al., 1973).

The effectiveness of intracoronary administration of GTN in patients refractory to GTN administered by other routes has been shown both in angiographically normal and atherosclerotic stenotic coronary arteries (Pepine et al., 1982). Intracoronary administration of GTN is used to treat coronary artery spasm, especially spasm induced during an ergonovine test, coronary catheterisation, or angioplasty. Intracoronary administration avoids systemic effects, including a possible sympathetic stimulation, and also results in a better approach of the drug to the spastic segment. Buxton et al. (1980) reported that intracoronary administration of GTN relieved ergonovine-induced coronary spasm refractory to other routes of drug administration.

Maximal coronary vasodilation has been observed with intracoronary GTN doses of 450 μg (Feldman et al., 1979, 1982). In another study, a near maximal effect was observed at doses of 200 μg, with only a slight additional dilatory effect at higher doses (Kern et al., 1986). Kern et al. (1986) observed a 53 ± 25% (mean ± SD)increase in coronary blood flow after intracoronary administration of 200 μg GTN. Liu et al. (1985) observed a 14% increase in mean coronary blood flow by 40 μg intracoronary GTN in nine patients.

The doses commonly used for intracoronary administration are too low to produce systemic hemodynamic effects. Feldman et al. (1982) found no change in heart rate after doses of 5 to 250 μg GTN, despite small reductions in systemic arterial pressure. The same applies to a study by Kern et al. (1986), in which doses of 50 to 300 μg produced reductions of 6% in mean systemic arterial pressure. Feldman et al. (1979) reported that 450 μg GTN resulted in an increase of five beats/min or more in heart rate and a decrease of more than 10 mm Hg in mean arterial pressure.

Intracoronary administration of GTN is commonly used as a part of routine PTCA. Dilation time is one of the determinants of the success rate of PTCA. It has been suggested that intracoronary injection of GTN can prolong dilation time and thus increase ischemic tolerance. Erbel et al. (1983) studied the effect of intracoronary GTN on dilation time in 10 patients with unstable angina pectoris and single vessel coronary disease. Two dilations were performed within 5 min, each time continuing until the development of angina pectoris or ventricular arrhythmias. GTN, 0.2 mg, was then injected into the coronary artery and dilation was repeated after 1.5 and 10 min. The drug increased ischemic tolerance in seven of 10 patients as evidenced by prolongation of dilation time to development of angina pectoris or ventricular arrhythmias.

Hermann et al. (1985) compared the effects of 0.2 mg GTN injected into the coronary arteries with effects of the same dose given intravenously in 66 patients undergoing coronary angioplasty. Both intracoronary and i.v. administration improved myocardial ischemia tolerance during angioplasty and, according to the authors, in both cases the effect was attributed to the systemic effects of the drug. This might suggest that routine intracoronary administration of GTN during angioplasty may not be necessary and that systemic administration is sufficient. Doorey et al. (1985) showed that a bolus dose of i.v. GTN (200 μg) diminished ischemic changes induced by balloon inflation used in the procedure of PTCA. The bolus dose was given 30 s before inflation. Recently, Johansson et al. (1990) showed, in a double-blind, randomised, placebo-controlled study, that 5 mg GTN given as a buccal formulation 30 min before the PTCA procedure significantly decreased myocardial ischemia during PTCA.

2. Isosorbide dinitrate and isosorbide-5-mononitrate. A continuous infusion of ISDN was administered to 12 patients with resting angina and severe coronary disease in a crossover study in a coronary care unit. The anginal attacks were associated with transient ST-segment elevations in 10 patients. All patients showed a beneficial response (Distante et al., 1979).

Oral ISDN (40 to 120 mg/day) was compared to nifedipine and placebo in two studies with a similar design (Ginsburg et al., 1982; Hill et al., 1982). Coronary artery spasm was documented in all cases. ISDN reduced the frequency of angina by 65% in both studies.

ISDN administered sublingually has been shown to relieve spasm resulting from ergonovine provocation (Bethge and Bachmann, 1983).

Intracoronary administration of ISDN was studied by Crake et al. (1987) in 19 patients during coronary angioplasty. They found that the duration of balloon inflation to the onset of ST-segment depression was prolonged 48% by the intracoronary administration of ISDN and that the duration to a 1-mm ST-segment depression was

prolonged by 28%. It might be concluded that ISDN delays the onset of myocardial ischemia during coronary angioplasty.

A study by Distante et al. (1985) indicated that 20 or 40 mg IS-5-MN is effective in the prevention of myocardial ischemia due to vasospasm induced by ergonovine or isometric stress testing.

C. Unstable Angina, Silent Myocardial Ischemia, and Myocardial Infarction

1. Unstable angina. Unstable angina may be simply defined as a rapid worsening of angina, i.e., newly developed (within the last 4 weeks) severe angina pectoris, increase in effort induced angina, or recent onset of angina at rest.

There have been only a few well-performed clinical trials of nitrates in patients with unstable angina, and they have involved small numbers. Mikolich et al. (1980) showed in an uncontrolled study that, in 40 of 45 patients with angina at rest, chest pain was effectively relieved by GTN infusion; similar results were demonstrated by DePace et al. (1982). In an uncontrolled study by Kaplan et al. (1983) it was shown that 33 of 35 patients with angina at rest were resistant to high doses of oral ISDN or cutaneous GTN, were relieved of pain ($n = 25$), or had pain ameliorated ($n = 8$) when increasing dosages of GTN were given intravenously. After GTN infusion for a few days, it is often possible to withdraw the treatment or change to oral or transdermal nitrates (Lin and Flaherty, 1985).

Horowitz et al. (1988) have shown that concomitant administration of i.v. GTN and N-acetylcysteine in patients with unstable angina pectoris may facilitate management by limiting the doses of GTN required and by reducing the incidence of acute myocardial infarction.

There is also some experience concerning i.v. ISDN in unstable angina. Distante et al. (1987) studied the effects of i.v. ISDN in 15 patients with unstable angina. When admitted to a coronary care unit these patients had angina at rest that was poorly controlled by oral and/or transdermal nitrates, calcium antagonists, and β-adrenoceptor blockers. Following ISDN infusion (mean dose 3.7 mg/h), episodes of myocardial ischemia were completely abolished in 11 of the 15 patients.

2. Silent myocardial ischemia. Silent myocardial ischemia, defined as asymptomatic ST-segment depression (ischemia without anginal pain) documented either by exercise tests or by long-term ECG recordings, seems to be a common feature in patients with known coronary artery disease. The prevalence of silent myocardial ischemia after a myocardial infarction, as assessed by exercise tests, has been estimated at 20 to 40% in several studies (Theroux et al., 1979; Sami et al., 1979; Markiewicz et al., 1977; Davidson and DeBusk, 1980). Studies of patients with angina pectoris have shown that about 60 to 80% of patients with stable effort angina also have frequent episodes of silent myocardial ischemia during Holter monitoring (Cecchi et al., 1983; Deanfield et al., 1984). These episodes seem to occur three to four times as often as painful ones (Deanfield et al., 1983, 1984).

ECG signs of myocardial ischemia appear to represent an important risk factor, both in symptomatic and asymptomatic subjects. Pharmacological intervention studies have shown that episodes of silent myocardial ischemia can be reduced, but there are no studies showing any prognostic impact.

Shell et al. (1986) studied eight patients with chronic stable angina pectoris and silent ischemia, as evident by ST-segment depression or elevation during ambulatory 24-h ECG monitoring. The patients were treated with β-blockers. Transdermal GTN was started at a dose of 5 mg/24 h, and the dose was increased by increments of 5 mg. At a mean dose of 10.4 mg/24 h, all eight patients had a decrease in the number of symptomatic events. The total number of silent and symptomatic ischemic events decreased from 5.3 to 0.8/24 h. Duration of ischemic events decreased from 95.8 to 17 min/24 h. The magnitude of ischemia, as estimated from the integral of ST-segment deviations, also decreased.

Pepine et al. (1986) demonstrated that, in patients with obstructive coronary artery disease, left ventricular dyskinesia can occur in the absence of symptoms or ECG evidence of transient myocardial ischemia and that dyskinesia improves in response to i.v. GTN. Global ejection fraction increased after i.v. administration of GTN, and this increase was concomitant with a reduction in left ventricular end-diastolic pressure. All dyskinetic anterior regions and 70% of inferior regions showed improved motion after i.v. GTN. The improvement in left ventricular wall motion occurred in the absence of any significant change in heart rate and in the presence of minimal changes in left ventricular systolic pressure. The findings suggest that GTN-induced improvement in wall motion probably resulted from enhanced perfusion to the ischemic myocardial segments.

Schang and Pepine (1977) reported beneficial effects of GTN on the frequency of silent myocardial ischemia. They used continuous 10-h ECG recordings, along with detailed diaries of activity and symptoms in 20 patients with coronary artery disease during a mean time of 16 months. Seventy-five percent of the episodes of ischemic ST-segment changes were silent and occurred at heart rates significantly lower than those occurring at the onset of angina during exercise testing. In 5 of the 20 patients, either GTN (0.4 mg) or placebo was administered sublingually at hourly intervals to assess the response to GTN. The frequency of asymptomatic episodes of ST-segment depression was significantly less with GTN (0.6 ± 0.2 episodes/monitoring period, mean $\pm$ SD) than with placebo (3.7 ± 0.2 episodes/monitoring period).

Continuous infusion of ISDN has been shown to decrease the frequency of episodes of silent myocardial ischemia in patients with vasospastic angina (Distante

et al., 1979). In this double-blind crossover study, the frequency of spontaneous asymptomatic ischemic ST-segment shifts decreased significantly during ISDN infusion.

In a controlled double-blind study, von Arnim and Erath (1988) used continuous Holter monitoring to compare the effect of IS-5-MN and nifedipine in patients with documented transient ischemic episodes. Seventy-five percent of the episodes were not accompanied by pain. Twenty patients with documented coronary heart disease were included; 15 finished the 4-week study. ST-segment deviation >1 mm for >1 min was considered as an ischemic episode. Patients received IS-5-MN (20 mg three times/day or 50 mg in a sustained-release tablet) or nifedipine (20 mg in a sustained-release tablet three times/day) in a random order during four 1-week periods. At the end of each week, Holter monitoring was repeated and showed reductions of episodes by 67 and 67% after the 2 weeks of IS-5-MN therapy and 56 and 58% after the 2 weeks of nifedipine therapy (all $P < 0.05$). Painful and painless episodes were reduced to a similar extent. Individual responses showed great variability, and in all treatment periods not more than half of the patients were completely free of ischemic episodes.

Feng et al. (1990) studied the anti-ischemic efficacy of IS-5-MN (20 mg three times daily) on silent myocardial ischemia after myocardial infarction in 28 patients; ambulatory ECG monitoring during 48 h was used in a randomised, crossover, single-blind, placebo-controlled fashion. The number of ischemic episodes decreased 88%, duration of ischemia was reduced by 94%, and total maximal ST-segment depression decreased 86%.

Whether the prognosis for an individual with silent ischemia is better if treated with nitrates that reduce the silent ischemic episodes requires further investigation.

3. Myocardial infarction. Animal and human studies have revealed that, in acute myocardial infarction, nitrates may reduce left ventricular filling pressure and even systemic vascular resistance. They may increase cardiac output, coronary blood flow, and contractility of ischemic segments and, in some cases, decrease infarct size. The use of nitrates in acute myocardial infarction in patients has, as yet, been studied mostly in trials with only a small number of patients, and few of the studies have been properly randomised and double blinded.

The relief of symptoms by GTN has been documented as reduction in the use of analgesics (Lis et al., 1984) and improvement of dyspnoea in acute left ventricular failure following acute myocardial failure (Gold et al., 1972; Flaherty et al., 1975; Korewicki et al., 1984). The hemodynamic effects of i.v. GTN infusions are well documented in acute heart failure.

GTN has been shown to enhance contractility of hypokinetic segments in the periphery of the ischemic zone (Theroux et al., 1976; Ramanathan et al., 1979; Banka et al., 1975). Shimoura et al. (1983) reported that in dogs the extent of enhancement of myocardial contractility was inversely related to the extent of ischemic damage.

The possible effect of GTN given intravenously on infarct size has been investigated in a number of studies, most of them including only a few patients, and the results are somewhat conflicting. Infarct size has been estimated by creatine kinase release, ECG indices, or ventricular wall motion analysis. The average dose of GTN has ranged from 40 to 90 μg/min.

Bussmann et al. (1981) demonstrated a significant reduction of creatine kinase release in patients irrespective of whether GTN was administered within or after 8 h of symptom onset. Korewicki et al. (1984) found that patients included within 8 h showed a reduction in creatine kinase release. Flaherty et al. (1983) did not show any reduction of creatine kinase in patients included within 12 h. In a subset of patients admitted to the hospital within 10 h, there was an increase in left ventricular ejection fraction and a decrease in infarct size as assessed by thallium 201 perfusion in the GTN group as compared to placebo. Jaffe et al. (1983) found a decrease in infarct size similar to that in the study by Bussmann et al. (1981) but only in patients with inferior infarcts. Derrida et al. (1978) and Jugdutt et al. (1983) showed a significant reduction of infarct indices in patients treated with GTN, preferentially patients with anterior infarction compared with controls, or patients with inferior infarcts.

In a study of a total of 310 patients, Jugdutt and Warnica (1988) investigated whether the effect that intravenously administered GTN has on creatine kinase release/infarct size during acute myocardial infarction is influenced by infarct location (anterior versus inferior) and timing (therapy <4 versus >4 h after onset of pain). Compared with controls, creatine kinase infarct size was less in the GTN group in both anterior and inferior infarction and both in early and late groups. Infarct-related major complications were less frequent in the GTN group than in the control group. Mortality was less in GTN than in control groups in the hospital (14 versus 26%, $P < 0.01$), at 3 months (16 versus 28%, $P < 0.025$), and at 12 months (21 versus 31%, $P < 0.05$). This advantage was, however, found only in anterior subgroups.

There are two other studies with smaller number of patients that have indicated a reduction in deaths as compared to placebo (Derrida et al., 1978; Bussmann and Haller, 1983). Yusuf et al. (1988) presented an overview of trials in which i.v. GTN or SNP had been given acutely and found that a typical reduction in the likelihood of death is approximately 35%. The greatest reduction in mortality seems to occur during the first week.

ISDN has been used especially to treat patients with congestive heart failure as a complication of a recent myocardial infarction. Both oral (Bussmann et al., 1977)

and i.v. administration (Rabinowitz et al., 1982; Nelson et al., 1983) have been used.

Cintron et al. (1988) reported a direct comparison of GTN and ISDN both administered intravenously to patients with acute myocardial infarction and elevated pulmonary wedge pressure. Blood pressure, heart rate, PCWP, cardiac output, medication dose in micrograms per minute, and recurring episodes were compared at baseline and at 6, 12, 18, and 24 h. Both drugs significantly reduced PCWP and increased cardiac output. The ISDN group required fewer retitration episodes and less increases in dosage than did the GTN patients at 24 h.

Studies of the possible influence of ISDN on infarct size have produced equivocal results. Osuna et al. (1985) studied patients with inferior infarcts and no effect was seen. Zaslavskaya et al. (1985) reported a reduction in creatine kinase in patients with myocardial infarction treated with ISDN. Grosser et al. (1983) reported a reduction in early deaths in patients with acute myocardial infarction treated with ISDN, 60 mg, orally.

In a randomised, double-blind, placebo-controlled multicentre trial (Fitzgerald and Bennett, 1990), oral IS-5-MN was tested in 360 patients with acute myocardial infarction. Prior to analysis, patients were stratified according to the presence or absence of left ventricular failure at the time of hospital admission. The trial therapy was given throughout 5 days. Overall mortality was 4.9% in the IS-5-MN group compared with 4.0% in controls after 5 days and 14.1% compared with 10.5% after 6 months (nonsignificant). A reduction in mortality in the IS-5-MN group with heart failure (IS-5-MN 7.9%, placebo 12.9% at 5 days) was found, in contrast with a nonsignificant increase in mortality in IS-5-MN-treated patients without heart failure (IS-5-MN 4.1%, placebo 2.1% at 5 days). The conclusion of the authors is that oral IS-5-MN can be of benefit in patients with acute myocardial infarction with heart failure, but they question the use of nitrates in acute myocardial infarction when heart failure is absent.

In conclusion, in the acute treatment of unstable angina and myocardial infarction, GTN given intravenously is of value in reducing symptoms (anginal pain and symptoms due to myocardial failure). Further studies are required to ascertain the effects of nitrates on complications and mortality.

D. Congestive Heart Failure

Organic nitrate esters were the first vasodilator drugs used to produce hemodynamic improvement in patients with ventricular failure (Johnson et al., 1957). The acute use of nitrates in heart failure complicating acute myocardial infarction was been discussed in the previous section; the long-term treatment of chronic congestive heart failure has been studied mostly in small patient populations.

1. Glyceryl trinitrate. Different dosage forms of GTN have been used in the treatment of congestive heart failure. Bussmann and Schupp (1978) studied sublingual administration of GTN (1.6 mg) and found that, within 10 min, left ventricular filling pressure decreased from 33 ± 10 to 24 ± 8 mm Hg (mean ± SD), and cardiac output increased from 3.3 ± 0.8 to 3.7 ± 0.8 liter/min. Dyspnoea, orthopnea, and pulmonary rales rapidly disappeared. However, in patients with massive peripheral edema, the effect of sublingual GTN might be dramatically impaired, as shown by Magrini and Niarchos (1980).

Buccal GTN has been investigated as well (Lahiri et al., 1984; Sanghera et al., 1986). Lahiri reported that 5 to 10 mg given three times daily (mean 23 mg/day) to 16 patients for 4 weeks resulted in significant increases in ejection fraction and exercise time.

There is at least one report concerning the effectiveness of oral sustained-release formulations of GTN (Amsterdam et al., 1979). Regarding a positive acute effect of the ointment formulation of the drug, there have been some reports (Awan et al., 1978; Taylor et al., 1976; Armstrong et al., 1980c).

Transdermal administration of GTN in the form of patches has been investigated in a number of studies. Different doses have been used and conflicting results have been obtained regarding the efficacy of the therapy. Sharpe and Coxon (1984) demonstrated a sustained drug effect up to 18 to 24 h with a low dose (5 mg/24 h); in long-term treatment (3 months), however, the reduction in left ventricular filling pressure was attenuated. In a randomised, within-patient, double-blind, placebo-controlled crossover trial, Bolognese et al. (1988) showed that a low dose (10 mg/24 h) resulted in a persistent effect on PCWP and cardiac index.

In a placebo-controlled, double-blind, crossover study, Lindvall et al. (1988) investigated the effect of transdermal GTN (5 to 15 mg/24 h) on 18 patients with moderate to severe congestive heart failure. Treatment periods were 4 weeks. Therapeutic effects were evaluated by clinical examination (New York Heart Association class), treadmill exercise, echocardiography, and subjective scaling of general well being, cardiac symptoms, and dyspnoea by the patients themselves and by the physician. When comparing values before and after each treatment period, only the investigator's evaluation of heart failure level proved significantly higher after the placebo period. No other findings of significance were observed. The authors concluded that transdermal GTN, given in doses of 5 to 15 mg/24 h during 4 weeks, fails to improve the clinical situation in patients with moderate to severe congestive heart failure.

Sharpe et al. (1987) tested transdermal GTN, 10 mg/24 h, on patients for 1 month; two different modes of application were used, 24 and 16 h/day. Initially, eight of 10 patients responded with a >20% reduction in mean pulmonary artery wedge pressure. Responders were randomised to intermittent or continuous treatment for 1 month, followed by 1 month of the alternate treatment.

After 1 month of intermittent treatment, the hemodynamic response after reapplication was similar to the initial response. After continuous treatment, hemodynamic values 24 h after application were similar to initial control values, and there was no change after reapplication. It was concluded that the moderate vasodilator effect of transdermal GTN in congestive heart failure is maintained with intermittent treatment, whereas continuous treatment resulted in the development of tolerance.

In other studies, much larger doses have been used to obtain an effect. In an investigation by Rajfer and coworkers (1984), the dose of transdermal GTN was estimated from an initial i.v. infusion of GTN; the mean dose was >30 mg/24 h, and six of the nine patients received 40 mg/24 h. A significant decrease of left ventricular filling pressure and a significant increase in cardiac index were noted, and the hemodynamic benefit persisted throughout 24 h. The authors remarked that "substantial doses of the drug may be required to produce a satisfactory hemodynamic response in most patients with congestive heart failure."

Packer et al. (1986) studied 22 patients with severe chronic heart failure and compared the effect of transdermal GTN with sublingual GTN and oral ISDN. Sixteen patients showed favourable hemodynamic effects with GTN patches, but the doses required varied greatly: 10 mg/24 h in six patients, 20 mg/24 h in five patients, 40 mg/24 h in three patients, and 60 mg/24 h in two patients. Of the six remaining patients, three did not respond to high dose transdermal GTN, even though marked effects were noticed after sublingual and oral nitrate administration; three others did not respond to any nitrate formulation by any route. Transdermal GTN produced rapid increases in cardiac index and decreases in right and left ventricular filling pressures, in mean arterial pressure, and in systemic vascular resistance ($P < 0.01$). These effects, however, became rapidly attenuated within 3 to 6 h. After 18 to 24 h, only modest decreases in right and left ventricular filling pressures could be observed.

Elkayam et al. (1985) investigated the effect of a high dose (90 mg) in 10 patients with severe chronic congestive heart failure. Hemodynamic measurements revealed a rather modest decrease in mean pulmonary artery wedge pressure. The change from baseline was significant only after 2 h.

2. Isosorbide dinitrate. ISDN has been used both in the acute and long-term treatment of congestive heart failure. Franciosa et al. (1974) reported that 20 mg of ISDN orally caused significant reductions in left ventricular filling pressure, persisting up to 4 h in patients with congestive heart failure. Williams et al. (1977) found a significant reduction in pulmonary artery wedge pressure after 20 mg of ISDN orally, and the effect persisted for 5 h. Franciosa and Cohn (1979) evaluated the effect of acute administration of ISDN on the response to submaximal and maximal exercise in patients with congestive heart failure. Maximal exercise capacity was not increased. In a later study, however, Franciosa et al. (1980) revealed that maximal exercise capacity increased after 3 months of treatment with ISDN. Franciosa and Cohn (1980) compared the effects of oral ISDN, 40 mg four times daily, and placebo, during 3 months in patients with chronic congestive heart failure. The initial decrease in PCWP induced by the drug was sustained in a repeated hemodynamic evaluation after 3 months. Leier et al. (1983) showed symptomatic improvement and an increase in exercise tolerance after 3 months of treatment with ISDN.

Oral ISDN, thus, has been widely used to decrease elevated left ventricular filing pressure in patients with chronic heart failure, and the recommended dose has usually been 40 mg every 6 h. However, according to clinical experience, some patients do not respond to the therapy. This was systematically studied by Kulick et al. (1988), in 50 patients with severe chronic heart failure due to left ventricular dysfunction. Twenty-seven (54%) of the patients responded to 40 mg of oral ISDN (>20% decrease in mean pulmonary artery wedge pressure sustained ≥1 h), and twenty-three patients (46%) failed to respond. Nonresponders had a significantly higher baseline right atrial pressure than responders did (14 ± 5 versus 10 ± 6 mm Hg, mean ± SD; $P < 0.02$). In addition, all seven patients with a baseline right atrial pressure of <7 mm Hg and 12 of 14 patients with a baseline right atrial pressure <10 mm Hg responded to 40 mg. Twenty-two of the 23 not responding to 40 mg received a higher dose (80 to 120 mg). Ten (45%) of these patients demonstrated the desired hemodynamic response, whereas 12 (25%) failed to respond to even a 120-mg dose.

ISDN lingual spray has been shown to give a rapid hemodynamic improvement in patients with congestive heart failure; a dose of 2.5 mg seemed to be optimal (Klein and Sharir, 1990). Decreases in right-sided pressures and an increase in cardiac output was observed within 1 min after administration and persisted for 20 to 30 min.

In a huge Veterans Administration study, 642 men with stable chronic congestive heart failure undergoing conventional treatment with digoxin and diuretics were randomly assigned to receive additional treatment with prazosin, both hydralazine and ISDN, or placebo. The trial was conducted double blindly. Left ventricular ejection fraction increased significantly at 8 weeks and at 1 year in the group treated with hydralazine and ISDN but not in the placebo or prazosin groups. Even more importantly, it was shown that ISDN in combination with hydralazine can reduce mortality; the doses used were 40 mg ISDN given four times per day and 75 mg hydralazine given four times per day. Mortality during the entire follow-up period was lower in the group that received hydralazine and ISDN than in the placebo group. For

mortality by 2 years, a major end point, the risk reduction among patients treated with both hydralazine and ISDN was 34% ($P < 0.028$). The mortality in the prazosin group was similar to that in the placebo group (Cohn et al., 1986).

3. Isosorbide-5-mononitrate. Experience in the use of IS-5-MN in patients with congestive heart failure is, as yet, limited. Mainly acute studies, often without proper control groups, have been performed.

Harf and Welter (1988) investigated the effect of IS-5-MN given intravenously to patients with cardiogenic pulmonary edema. The therapy consisted of a bolus dose (13.45 ± 8.14 mg, mean ± SD) followed by an infusion (mean 0.12 mg/kg/h). The results were evaluated by clinical data, blood gases, and chest radiographs; most patients experienced a fast respiratory relief. Gammage et al. (1986), treating patients with acute left ventricular failure following acute myocardial infarction, used oral IS-5-MN after an initial challenge with 5 mg IS-5-MN i.v. as a bolus. Thereafter, the patients were given 20 mg orally every 8 h for 48 h. Following i.v. IS-5-MN, PCWP decreased from 26.2 to 17.5 mm Hg and remained at an acceptable level throughout the entire 48-h period (13.1 mm Hg at 48 h). In a study in which IS-5-MN and GTN were compared, both given intravenously, Schneeweiss (1988) showed that PCWP was reduced from 31.5 to 15 mm Hg by IS-5-MN, with a reduction of mean arterial pressure from 89.2 to 79.8 mm Hg. GTN gave a lesser reduction of PCWP (to 19.6 mm Hg) but a more pronounced decrease in mean systemic arterial pressure (to 67.5 mm Hg).

There are also some studies showing that IS-5-MN, when administered intravenously (Rabinowitz et al., 1988; Hutton et al., 1987) or orally (Rubartelli et al., 1987; Lopez Sendon et al., 1987; Schiavoni et al., 1985; Tronconi et al., 1985), has an acutely favourable effect on patients with congestive heart failure. However, all of these studies lack a proper control group. Obviously, well-controlled studies are needed to properly evaluate IS-5-MN in congestive heart failure, especially with regard to long-term treatment.

In conclusion, GTN or ISDN administered sublingually or intravenously can be used for the acute treatment of severe congestive heart failure. For chronic treatment, there is, at present, documentation only for oral administration of ISDN. The possible effect of ISDN on mortality should be studied further.

E. Miscellaneous Therapeutic Uses of Nitrates

1. Peripheral vascular diseases. As early as 1948, Lund reported that the use of GTN ointment produced symptomatic relief in patients with peripheral circulatory disorders. Approximately 30 later, Francis et al. (1977) studied the effect of GTN ointment on plethysmographic measurements in the lower extremities. These authors found an increase in pulse volume, not only in the foot where the ointment was applied but also in the contralateral foot, although less prominently, indicating a local effect on the circulation as well as a systemic effect. Coppock et al. (1986) studied the response to topical GTN by digital plethysmography in 17 patients with Raynaud's phenomenon. Improvement was significant in those in whom the disease was secondary to an underlying connective tissue disorder.

Testa and coworkers (1988) studied the effect of ISDN ointment (100 mg three times daily) applied directly to the areas where ischemic pain was experienced, in 30 male patients with stable documented intermittent claudication. Initially, and after 1, 3, 6, and 12 months, treadmill stress tests were conducted and the symptom-free distance walked and the maximum distance reached were evaluated. The evaluation of the first month was double blind, half of the patients receiving placebo. ISDN was significantly better than placebo. Thereafter, all patients were treated with ISDN in an open study and, on all occasions of evaluation, significantly better results were reached compared with the basal value.

2. Noncardiovascular uses. Nitrates relax all kinds of smooth muscles, not only vascular smooth muscle, a fact that has allowed the use of these drugs to treat many different ailments.

Nitrates have, for example, been utilised in the treatment of oesophageal spasm (Orlando and Bozymski, 1973). Gelfond et al. (1981) and Rozen et al. (1982) have shown the beneficial effect of sublingually administered ISDN on patients with achalasia. Nitrates have also been used to treat spasm in hepatic ducts and the urinary tract and have been shown to lower portal pressure (Freeman et al., 1985; Hallemans et al., 1983; Navasa et al., 1989).

In patients with open-angle glaucoma, orally administered ISDN significantly lowered the intraocular pressure (Wizemann and Wizemann, 1980). Topical application of GTN has been used as an aid in inserting peripheral venous catheters in neonates (Hecker at al., 1983; Maynard and Oh, 1989) and in reducing infusion phlebitis (Khawaja et al., 1988). GTN and ISDN have been shown to relax penile tissue in vitro (Heaton, 1989) and topical GTN has been shown to increase blood flow and diameter in the cavernous arteries of impotent men (Owen et al., 1989).

3. Airways diseases. A century ago, Osler (1892) considered nitro compounds to be efficacious in treating asthma. They fell into disuse, however, after the discovery of the usefulness of adrenergic agonists. Nitro compounds such as GTN and pentaerythritol tetranitrate have been reported to produce bronchodilation in animal studies in vitro as well as in vivo (Barlow and Beams, 1933; Aviado et al., 1969). Moreover, Byrick et al. (1983) demonstrated that GTN, given intravenously, relaxed large airways in humans. In clinical studies of patients with chronic obstructive lung disease, forced expiratory volume in 1 s was found to increase after sublingual GTN

administration (Hirschleifer and Arora, 1961; Von Peter, 1980). Miller and Shultz (1979) found GTN to be ineffective as a bronchodilator in patients with clinically stable asthma. Sublingual GTN has also been tested in acute asthma; no significant change in forced expiratory volume in 1 s and forced vital capacity was observed following sublingual GTN, and some of the patients, instead, experienced transient but severe hypotension (Kennedy et al., 1981). The results of these studies suggest that sublingual GTN is not adequate initial therapy for asthmatic attacks and that the sublingual administration of GTN to patients with acute asthma may actually be dangerous (Kennedy et al., 1981; Goldstein, 1984).

Attempts have also been made to reduce severe pulmonary hypertension secondary to respiratory failure; in open studies GTN was administered intravenously to some patients with pulmonary hypertension. This treatment resulted in a decrease in pulmonary artery resistance and an increase in forced expiratory volume in 1 s and cardiac output (Lappe et al., 1977; Fourrier et al., 1982).

There are two major potential complications of vasodilator therapy in pulmonary hypertension, systemic hypotension and intensification of hypoxemia which can be fatal (Rubin, 1983).

In conclusion, although nitro compounds are potent relaxants of smooth muscle in several tissues, clinical usefulness seems to be limited to the cardiovascular system.

VIII. Tolerance Development and Dependency

Tolerance to a drug exists when, after repeated administration, increasing dosages are required to continue to obtain a given pharmacological or therapeutic effect; tolerance disappears after cessation of exposure to the drug. Cross-tolerance may occur among related compounds.

Since the end of the last century, many reports have been published concerning the diminishing efficacy of organic nitrates during long-term use in the treatment of different cardiovascular diseases or in experimental studies. However, only during the last decade has tolerance to organic nitrates been more systematically evaluated in clinical settings. In such evaluations (i.e., when considering tolerance against the antianginal effect/anti-ischemic effect), the methods used are of utmost importance as are the number of and selection of patients. As will become evident in the following section, there are discrepancies among different studies regarding whether tolerance develops or not; such discrepancies can occasionally even exist between studies performed using the same drug, the same formulation, and the same dosing regimen. There are a number of reasons for this, one of significance being study design. Studies should not be open but should be randomised, double blind, and, if possible, placebo controlled. A sufficient number of patients should be included to allow detection of clinically important differences. Studies should be performed with the actual patient group for which the drug is intended. The outcome criteria should be clearly defined and the outcome measures appropriate for the objective. Usually some kind of exercise test is included to evaluate the anti-ischemic effect. It is of utmost importance that the test is performed at a reasonable time after dosing, because the development of tolerance shortens the duration of effect. Proper investigation of tolerance development necessitates an exercise test at the end of the intended dosing interval, which has usually only been the case in studies using transdermal patches. It is remarkable that for formulations intended for oral administration more than once daily, there was only one study (Bassan 1990) that attempted to evaluate the anti-ischemic effect after the second (or third) dose during a day (i.e., toward the end of the interval covered by the nitrate).

There seem to be huge differences among different patients regarding the risk of developing tolerance. Therefore, it is of value that every patient in a study is described individually and also that the results of the exercise test and other investigations are presented for each individual. In addition, it is important to have reasonable control of patient compliance, because the ingestion of every tablet and ingestion at the correct time is essential when drawing conclusions regarding tolerance development.

A. Tolerance Development

1. Glyceryl trinitrate. The introduction of transdermal delivery systems (patches) for GTN stimulated a number of clinical studies of their long-term effects, because the suspicion arose early that the effect of a patch was attenuated within 24 h, despite a sustained plasma concentration, and was, thus, subject to the development of tolerance. Compared with other forms of administration, GTN patches have been well studied regarding development of tolerance. Regarding sublingual administration of GTN, no report has indicated tolerance development. The effect is very short-lived, and the plasma concentration rapidly decreases.

a. INTRAVENOUS GLYCERYL TRINITRATE. In a placebo-controlled study, Zimrin et al. (1988) evaluated the effect of an infusion of GTN during 24 h with repeated exercise tests. Compared to placebo, treadmill exercise performance during GTN infusion was improved after 1, 4, and 8 h, but no effect was seen at 24 h despite constant plasma levels. During the 24-h infusion, there was a progressive reduction in exercise duration to the antianginal endpoints.

In congestive heart failure, an attenuation of the initial beneficial hemodynamic effects has been observed within 24 to 48 h (Elkayam et al., 1987; Packer et al., 1987). Treating 35 patients with severe chronic heart failure, Packer et al. (1987) used a prolonged (48 h) i.v. infusion

of GTN (6.4 μg/min/kg), given either continuously or intermittently (12-h infusions separated by intervals of 12 h). Intravenous GTN produced immediate hemodynamic benefits in all patients, but this improvement was greatly diminished after 48 h of continuous therapy. This attenuation was accompanied by cross-tolerance to oral ISDN. In contrast, intermittent therapy with i.v. GTN was not associated with a loss of hemodynamic effect or cross-tolerance to oral ISDN. In eight patients who had developed nitrate tolerance during continuous therapy, the administration of N-acetylcysteine (200 mg/kg orally) restored the hemodynamic state to that observed at the start of the infusion of GTN (partial reversal of tolerance). The dose used in this investigation was very high. Studying patients with acute myocardial infarction, Jugdutt and Warnica (1989) found indications of tolerance development even using low dose regimen.

In a placebo-controlled, double-blind trial with intraindividual changes in a randomised sequence, 12 patients received either GTN (3 mg/h) or placebo via an infusion system, continuously for 24 h. The ischemia reaction in the exercise ECG (sum of ST-segment depressions) 90 min after onset of the infusion was improved by a mean of 45% during GTN treatment compared with the placebo. At the end of the 24-h period of infusion, however, the significant anti-ischemic effect was no longer demonstrable (Schneider et al., 1988).

b. BUCCAL AND ORAL GLYCERYL TRINITRATE. The transmucosal controlled-release tablet is a relatively new formulation that can be used both for aborting an angina pectoris attack and for prophylactic purposes. This is possible because of the prompt (60 to 90 s) onset of action and a duration of action of 4 to 5 h (i.e., until the tablet is completely dissolved). There is, as yet, no report of tolerance development in patients treated with buccal GTN. The reason for this is most probably that the formulation has been used as was intended, namely, for situational prophylaxis: the patient uses the drug only when performing activities that usually provoke an anginal attack, and this leads to nitrate-free intervals. Parker et al. (1985) made a direct comparison of the long-term efficacy of buccal GTN and oral ISDN. They used 3 mg buccal GTN three times daily and 30 mg ISDN four times daily. Treadmill exercise time was measured 1, 3, and 5 h postdosing on days 1 and 14. Both formulations significantly increased exercise time after the first dose. However, after 14 days of therapy, the effect of ISDN was superior to placebo only at 1 h after dosing, whereas the effect of buccal GTN was maintained. The authors drew the conclusion that the dosing regimen with administration only three times daily gave a sufficient nitrate-free interval to prevent tolerance development for buccal GTN but not for ISDN given four times daily.

No studies have been specifically designed to evaluate the development of tolerance for GTN administered as sustained-release formulations for oral administration. The question of tolerance was, in the study by Winsor and Berger (1975), addressed by counting the consumption of sublingual GTN every week during an 8-week treatment period; the consumption of GTN was not higher at week 8 as compared to week 1.

Berkenboom et al. (1984) studied the effect of sustained-release GTN, 6.5 mg three times daily, in 46 patients in a randomised placebo-controlled double-blind trial over 2 weeks. They found a slight, but statistically significant, decrease in the number of anginal episodes and in the consumption of sublingual GTN. An exercise test 1 h postdosing showed an increase in exercise capacity expressed as exercise duration; symptom-limited exercise ST depression was significantly decreased during GTN therapy. However, an exercise test 1 h after dose administration has only a very limited value in evaluating the development of tolerance.

c. OINTMENT AND PATCHES. The development of tolerance during therapy with GTN ointment has not been properly studied. Sustained antianginal effects during long-term treatment have been reported (Reichek et al., 1974). A number of studies have clearly shown that continuous use (24 h application) of transdermal GTN patches gives an attenuated effect, both hemodynamically and with regard to its anti-ischemic effect. The development of tolerance is a clinical problem both in angina pectoris therapy and in the treatment of congestive heart failure. Patches represent the only formulation of GTN that has been properly studied regarding tolerance development (with the possible exception of the i.v. formulation); the hemodynamic and antianginal effects have been studied over the entire dosage interval, i.e., 24 h.

Soon after the introduction of GTN patches, reports appeared indicating that the antianginal effect did not persist for 24 h (Reichek et al., 1983; Crean et al., 1983). This was confirmed by several studies within the next few years (Parker and Fung, 1984; Reichek et al., 1984; Thadani et al., 1986; Sullivan et al., 1985), and the explanation was found to be a rapid (within 24 h) development of tolerance. Other studies have shown that the plasma concentration of GTN is maintained for 24 h after application (Muller et al., 1982; Colfer et al., 1982).

A meta-analysis of 17 randomised clinical trials of transdermal GTN systems (Colditz et al., 1988) and a Food and Drug Administration-initiated multicentre trial of transdermal GTN use for the treatment of angina pectoris (Abrams, 1989), using doses from 15 to 105 mg/24 h versus placebo, both show that with long-term or daily use of GTN the beneficial first dose antianginal effect was lost. This effect occurred only at the 4-h postdose exercise test on the first day of therapy and was not present 24 h postdosing.

Intermittent GTN therapy has been used in an attempt to obtain a persistent antianginal effect during

long-term administration. Cowan et al. (1987a,b) found that 12-h daily intermittent therapy resulted in a sustained effect after 1 week, whereas continuous (24 h) treatment gave an attenuated effect.

Schaer et al. (1988)and Luke et al. (1987) showed that intermittent therapy remained effective, but continuous therapy did not.

However, there have been studies that have indicated development of tolerance despite intermittent therapy. In a randomised, placebo-controlled trial in 36 patients with stable angina, continuous and intermittent (18 or 14 h/day) treatment with 10 mg/day was compared (Waters et al., 1989). Exercise tests were performed during the last 2 h of patch application. Compared with placebo, neither continuous GTN nor the two intermittent regimens prolonged total treadmill time or time to 1-mm ST-segment depression.

Nabel et al. (1989) used continuous ambulatory ECG monitoring to investigate anti-ischemic efficacy and development of tolerance to transdermal GTN. Patients demonstrated initial hemodynamic responsiveness to sublingual GTN and were titrated to a maximally tolerated dose of 30 to 60 mg/24 h. Two crossover phases were used in a randomised, double-blind, placebo-controlled manner, and continuous (24 h) and intermittent (12-h active drug followed by a 12-h nitrate-free period) GTN therapy were examined. Reductions in frequency and duration of ischemic episodes were present on day 1 of continuous therapy, but ischemic episodes returned to placebo levels by day 2, suggesting the development of tolerance. Even intermittent therapy was unable to prevent the development of tolerance on day 2 of treatment.

Schirnick and Reifart (1989) investigated a new patch designed to prevent development of tolerance. The patch had a GTN dose of 15 mg and a drug release of 70% during the first 12 h of application. Twelve patients with angiographically significant coronary artery disease, angina, and reproducible ST-segment depression were studied during exercise in a double-blind placebo-controlled trial. Stress tests were performed before and 1, 4, and 8 h after the first day application and again after 8 to 10 days of therapy. It was found that the patch was effective between 1 and 8 h of acute application. With chronic therapy, however, tolerance occurred despite the fact that only a small amount of GTN was released during the last half of a 24-h application.

The development of tolerance to GTN has been shown in congestive heart failure as well. Sharpe and Coxon (1984) demonstrated a sustained effect on PCWP for up to 24 h following a low dose of 5 mg/24 h, whereas a significant reduction in systemic vascular resistance and an increase in cardiac and stroke volume indices occurred on the first day at 4 h but were not maintained. In long-term treatment (3 months), the reduction in left ventricular filling pressure was attenuated, although still significantly reduced compared with the basal value. In a randomised and within-patient crossover trial that was also double blind and placebo controlled, Bolognese et al. (1988) showed a persistent effect on PCWP and cardiac index with a low GTN dose (10 mg/24 h). They propose that this persistent effect is due to the low dose and that a high dose would be more likely to induce tolerance.

Sharpe et al. (1987) tested 10 mg/24 h of transdermal GTN in patients for 1 month with two different modes of application (24 h/day and 16 h/day). After 1 month of intermittent treatment, the hemodynamic response after reapplication was similar to the initial response, whereas after continuous treatment, hemodynamic values 24 h after application were similar to initial control values, and there was no change after reapplication. Packer et al. (1986) studied 22 patients with severe chronic heart failure and compared the effect of transdermal GTN with sublingual GTN and oral ISDN. Transdermal GTN produced rapid increases in cardiac index and decreases in right and left ventricular filling pressure, mean arterial pressure, and systemic vascular resistance ($P < 0.01$). These effects, however, became rapidly attenuated within 3 to 6 h; after 18 to 24 h, only modest decreases in right and left ventricular filling pressures were observed.

2. *Isosorbide dinitrate.* Numerous well-designed clinical trials have demonstrated the development of clinically significant tolerance after long-term ISDN administration to patients with stable angina pectoris (Thadani et al., 1980, 1982; Parker et al., 1983). In the study by Thadani et al. (1982), 12 patients with stable angina were studied following 1 week of therapy with various doses (30, 60, or 120 mg four times daily). The antianginal effects were evaluated by exercise test at various times after drug administration. The measurements were performed on both the first and the last day of treatment. The time to moderately severe angina was increased by up to 8 h after dose administration on the first day compared with a significant increase of up to only 2 h after dosing on the last day of the trial. The effects studied were thus diminished in magnitude and duration, but an effect at 2 h was still present.

Similar results were presented in a placebo-controlled, double-blind study of patients with stable angina pectoris treated with a sustained-release preparation of ISDN (Blasini et al., 1980). In addition to hemodynamic effects, the antianginal effect of ISDN was evaluated by determination of ST-segment depression and exercise capacity during tests on a bicycle with an ergometer. The tests were performed after acute administration of 20 to 60 mg ISDN and after 8 weeks of chronic treatment with 20 to 40 mg ISDN three times daily. The hemodynamic and antianginal efficacy of ISDN, which was clearly present after acute administration in a dose-dependent manner, could no longer be observed after chronic treatment. Neither nitrate consumption, nor the rate of an-

ginal attacks, nor the exercise capacity and ST-segment depression differed significantly between the ISDN group and the placebo group.

Parker et al. (1985) found, in a comparison with buccal GTN, that 30 mg ISDN administered orally four times/day gave an attenuation of the antianginal effect during 14 days of treatment. In a later study Parker et al. (1987a) studied the development of tolerance to ISDN in 12 patients with chronic stable angina. The effect of 30 mg ISDN on treadmill exercise performance was assessed before a single dose and 1, 3, and 5 h after the dose. As compared to placebo, during the 5-h observation period the drug increased treadmill walking time prior to the onset of angina. The patients then received 30 mg ISDN twice, three times, and four times daily for a period of 1 week and thereafter exercise performance was assessed before and at 1, 3, and 5 h after the final morning dose. During sustained treatment two and three times a day, treadmill walking time was longer throughout the 5-h testing period than during the placebo phase. In contrast, during treatment four times daily, treadmill walking was prolonged at 1 h but not at 3 and 5 h after the last dose.

The influence of dose and administration schedule was discussed by Rudolph et al. (1983), who studied two groups of patients treated with different administration schedules of ISDN. Signs of tolerance development were seen in patients receiving 40 mg, four times/day, whereas in patients treated with 20 mg, two times/day, antianginal effect was completely maintained during the chronic phase of treatment.

In a study by Parker et al. (1984), 12 patients with chronic stable angina pectoris underwent treadmill exercise testing before and during a 24-h period after application of 100 mg of transdermal ISDN and matching placebo. Treadmill walking time to the onset of angina and to the development of moderate angina was significantly prolonged 2, 4, and 8 h after drug application but not after 24 h. The patients received the same treatment regimens for 7 to 10 days and underwent repeated exercise testing. During this sustained phase, treadmill walking time to onset of angina and to development of moderate angina was similar 4, 8, and 24 h after application of ISDN and placebo. Transdermal ISDN in a dose of 100 mg seems to be effective for 8 h during acute therapy, but during sustained therapy tolerance develops and no antianginal effect persists (Parker et al., 1984).

A number of studies have evaluated ISDN in the treatment of congestive heart failure. Franciosa and Cohn (1979) evaluated the effect of acute administration of ISDN on the response to submaximal and maximal exercise in patients with congestive heart failure. Maximal exercise capacity was not increased. In a later study, however, Franciosa et al. (1980) found that maximal exercise capacity increased after a 3-month treatment with ISDN. The interpretation of the study is complicated by the fact that the long-term effects of ISDN on hemodynamic variables were assessed 14 h after the last dose of the drug, which may have reversed any tolerance that had developed during long-term treatment.

Franciosa and Cohn (1980) compared the effects of oral ISDN, 40 mg four times daily, and placebo in patients with chronic congestive heart failure during 3 months. The initial decrease in PCWP induced by the drug was sustained in repeated hemodynamic evaluation after 3 months. Leier et al. (1983) showed symptomatic improvement and an increase in exercise tolerance after 3 months of treatment with ISDN. However, an attenuation of the effect on the arterial resistance vessels was noticed after 3 months of therapy.

3. Isosorbide-5-mononitrate. A number of studies have demonstrated that a 20-mg twice a day dose of IS-5-MN has a persistent antianginal activity during long-term treatment (Muller et al., 1983a; Akhras et al., 1985; Rennhak et al., 1985; Mefert and Paeckelmann, 1987; Uberbacher et al., 1983). Also, sustained-release formulations of IS-5-MN, intended to be administered only once a day, are available. Nyberg et al. (1986) showed in a double-blind crossover study against placebo that 60 mg IS-5-MN in a controlled-release formulation given once daily significantly increased exercise tolerance until the onset of angina and until 1-mm ST-segment depression; this occurred both after the first dose and after 1 week of treatment. In a recent study by Wisenberg et al. (1989), sustained-release IS-5-MN, 60 mg given once daily, produced a sustained antianginal effect during 11 to 14 days of treatment.

There are, however, a number of studies showing attenuation of the hemodynamic effect of both plain and sustained-release formulations of IS-5-MN. Jansen et al (1982) showed that both the hemodynamic and antiischemic effect of IS-5-MN declined when the drug was administered in a dose of 50 mg three times/day, whereas a dose of 20 mg three times/day did not lead to tolerance development.

In a placebo-controlled, double-blind, randomised trial, Kohli et al. (1986) used computerised exercise testing to evaluate the acute and sustained effect of IS-5-MN in 18 patients with stable angina pectoris. Acute testing was performed 2 h after the first dose and 2 h after the morning dose on day 14 when the patients had been treated with 40 mg twice daily for 2 weeks. The drugs were administered every 12 h. Acute testing showed an increase in exercise time after a single dose of IS-5-MN. Time to 1-mm ST-segment depression increased significantly, and peak exercise ST-segment depression decreased significantly. After 2 weeks of therapy, exercise time was similar for chronic placebo and for chronic IS-5-MN treatments. However, time to 1-mm ST-segment depression was significantly greater after chronic IS-5-MN. The authors concluded that the data suggest an attenuation of effect with respect to exercise time but a sustained beneficial effect on the ST-segment variables.

It is also noteworthy that five of the 15 patients who completed the study maintained the beneficial acute effect on exercise time.

Thadani et al. (1987b) studied the duration of effects of 20 and 40 mg IS-5-MN and placebo in patients with angina pectoris after the first dose and after 1 week of twice-daily therapy. The study was double blind, randomised, and crossover in design. Compared with placebo, after the first dose of IS-5-MN, exercise duration was longer at 2 and 6 h postdosing. After 1 week of twice-daily therapy, exercise duration increased at 2 h after the dose but not at 6 or 10 h. Tolerance to the antianginal effects developed during twice-daily therapy with 20 and 40 mg of IS-5-MN despite higher plasma IS-5-MN concentrations after 2 and 6 h during twice-daily therapy than after the first dose. The tolerance was characterised by a reduced peak effect after 2 h and a shortened duration of action compared with first dose effects.

In another study by Thadani et al. (1987a), the duration of effects of 50 and 100 mg of slow-release IS-5-MN was evaluated after the first dose and after once-daily therapy for 1 week in patients with stable angina pectoris. The study was randomised, double blind, and placebo controlled. After the first dose of 50 or 100 mg of slow-release IS-5-MN, the exercise time to the onset of angina and total exercise duration increased at 4 h but not at 20 or 24 h. After once-daily therapy for 1 week, no improvement in exercise duration or reduction in ST-segment depression was seen after 50 or 100 mg IS-5-MN at 4, 20, or 24 h, despite high plasma IS-5-MN concentrations.

It has been advocated that, provided a sufficiently long "nitrate-free" interval is obtained, the development of tolerance can be avoided. Hughes et al. (1989) studied asymmetrical dosage timing of IS-5-MN. In a placebo-controlled, double-blind, randomised study of 19 patients with stable chronic angina, the effect of IS-5-MN, 40 mg given twice daily (8 a.m..and 2 p.m.), was evaluated using computerised exercise testing. Acute testing was performed 2 h after the first dose and chronic testing 2 h after the morning dose on day 14. Acute testing showed an increase in exercise time from 6.7 to 10.1 min ($P < 0.01$) after a single dose of 40 mg IS-5-MN. The time to 1-mm ST-segment depression increased significantly during acute testing with IS-5-MN. After 2 weeks of therapy, exercise time was not different from placebo, and the improvement in ST-segment variables were not sustained. Thus, despite an asymmetrical dosage regimen, tolerance developed.

Wisenberg et al. (1989) compared the effect of a single 60-mg daily dose of a controlled-release preparation of IS-5-MN with 30 mg of ISDN given four times daily in a double-blind, randomised, placebo-controlled crossover study of 18 patients with chronic stable angina pectoris. The comparisons were carried out on the first day of therapy and after 11 to 14 days of continuous therapy to assess the duration of effectiveness and the development of tolerance. In short-term therapy, both drugs produced a significant improvement in treadmill walking time to moderate angina in comparison with placebo. The slow-release IS-5-MN was administered at 8.30 a.m. and the last exercise test was performed at 8.30 p.m. After 11 to 14 days of sustained therapy, IS-5-MN retained a beneficial effect on exercise time 4 and 8.5 h postdosing but not 12 h postdosing, whereas ISDN did not produce a significant increase at any time point. The authors concluded that 60 mg of slow-release IS-5-MN provide a significant antianginal effect, with both short-term and sustained therapy for at least 8.5 h after a single morning dose.

The question of the importance of different dose regimens on the development of tolerance and the possible association with plasma levels was recently addressed by Wagner et al. (1990). They gave three different dose regimens of a controlled-release formulation of IS-5-MN (60 mg/tablet) to healthy male volunteers. Dose regimen I consisted of a single daily dose of 60 mg given for 5 days. Dose regimen II started with a dose of 60 mg, followed by 30 mg 12 h later, and thereafter every 8 h; the last dose on the 5th day was again 60 mg. In dose regimen III, 60 mg followed by 30 mg 6 h later was administered every day for 5 days. The peripheral arterial and venous effects of IS-5-MN were monitored by changes in the finger pulse curve, standing systolic blood pressure, heart rate, and venous distensibility. Plasma concentrations of IS-5-MN were measured frequently following the first and last dose. Following dose regimen I, all hemodynamic effects produced by the first dose were maintained during the study. The maximal plasma concentrations were about 400 ng/ml, and the trough value was lower than 100 ng/ml. Following dose regimen II, the hemodynamic effects of IS-5-MN and sublingually administered GTN were completely abolished on the 5th day. Trough plasma concentrations were approximately 300 ng/ml during the entire study period. Following dose regimen III, pronounced hemodynamic effects were seen on the first day. However, a significant attenuation was seen on the 5th day, when trough plasma concentrations were between 100 and 230 ng/ml. The authors concluded that the maintenance of plasma concentrations of 300 ng/ml or higher produces rapid development of hemodynamic nitrate tolerance, whereas no tolerance development was found when the plasma concentrations were allowed to decline below 100 ng/ml before the next dose was given. In contrast, however, Thadani et al. (1987a) found a complete hemodynamic and antianginal tolerance when a slow-release formulation of 50 mg IS-5-MN was administered once/day for 1 week, although the minimum and maximum plasma concentrations were in the same ranges as those obtained in dose regimen I in the study by Wagner et al. (1990).

The possible development of tolerance during IS-5-

MN treatment of congestive heart failure has not been studied.

B. Cross-tolerance

In a now classical report by Schelling and Lasagna (1966), it was demonstrated that the effect of sublingual GTN on blood pressure and heart rate was diminished, but not abolished, during treatment with pentaeritrityl tetranitrate during 4 weeks. Zelis and Mason (1975) studied healthy volunteers and found that sublingual GTN induced a decrease in blood pressure and also caused arterial and venous dilation, both before and 6 to 8 weeks after administration of 120 mg ISDN in slow-release preparations. The blood pressure and the arterial vascular resistance decreased to the same extent in the control and during the ISDN period, whereas the small increase in venous volume induced by GTN was totally abolished during the ISDN period. Cross-tolerance between ISDN and GTN was also observed by Thadani et al. (1980); studying patients with coronary heart disease, these authors compared the dose-response curves for the nitrate-induced decrease in blood pressure after acute and sustained ISDN therapy (5 days, 15 to 60 mg four times daily).

In a randomised, double-blind, crossover study, 10 patients with stable exercise-induced angina pectoris were studied during acute and sustained therapy with oral ISDN (Dalal et al., 1983). Circulatory changes and exercise performance were evaluated before and 2 and 6 h after medication. Sublingual GTN was administered 30 min after the 2- and 6-h exercise tests, and the exercise test was repeated after another 5 min. The authors concluded that there is a possible cross-tolerance between ISDN and GTN due to a reduced effect of GTN in the ISDN-treated group.

Venous capacitance was assessed by Manyari et al. (1985) using the radionucleide blood pool method to determine whether cross-tolerance to sublingual GTN develops in the venous system when patients are taking oral ISDN. Before therapy with ISDN, sublingual GTN (0.6 mg) decreased systolic blood pressure by 14% and increased heart rate by 17%, and regional blood volume increased to 111% at 5 min relative to baseline measurements. During chronic therapy with ISDN, systolic blood pressure decreased by only 7% after GTN, and heart rate increased by 7%, whereas regional blood volume was 103% at 5 min after GTN. The changes in blood pressure, heart rate, and regional blood volume after GTN treatment during ISDN therapy were all significantly less than those observed before therapy with ISDN. The authors concluded that partial, but significant, cross-tolerance between ISDN and GTN develops in both the arterial and venous systems.

No studies have shown that in clinical practice one in particular of the three different organic nitrate esters used (GTN, ISDN, and IS-5-MN) has any advantage regarding development of tolerance; all of the substances have been shown to induce tolerance.

C. Differences between Different Target Organs

Numerous investigations have demonstrated the development of tolerance to GTN induced in vitro. This applies to both arteries (Axelsson et al., 1982; Ahlner et al., 1987a; Rösen et al., 1987), including coronary arteries (Torresi et al., 1985), and veins (Ahlner et al., 1986b; Rösen et al., 1987). Rösen et al. (1987) directly compared arteries and veins from the femoral and mesenteric regions in rabbits.

There has been no general agreement concerning the susceptibility to tolerance of the venous and arterial systems in vivo. Some groups have reported that tolerance occurs more often in the venous system (Zelis and Mason, 1975; Sutton and Fung, 1983), and others have demonstrated the opposite (Parker et al., 1983; Leier et al., 1983). In dogs Stewart et al. (1986) assessed venous tone as total effective vascular compliance in the non-tolerant state and during a 5-day infusion of GTN (1.5 μg/kg/min). During long-term treatment, baseline total effective vascular compliance was unaffected, and the GTN dose-response relationship for total effective vascular compliance was shifted to >10-fold higher doses, whereas baseline mean arterial pressure was lowered by 17 ± 3 mm Hg (mean ± SD) without any shift in GTN responsiveness. This lowering of mean arterial pressure was observed only after autonomic blockade. The authors concluded that long-term exposure to GTN results in tolerance to its venodilating effects, whereas arteriolar action is maintained.

Stewart et al. (1987) studied the effect of GTN tolerance on large coronary artery dilation in dogs. With long-term GTN (1.5 μg/kg/min i.v. for 5 days) treatment, the diameters of the left circumflex and anterior descending coronary arteries showed an initial increase of 8.2 ± 0.3% and 10.8 ± 0.9%, respectively, returning to control levels by the second to third day of treatment. On days 4 and 5, the dose-response relationships for GTN-induced epicardial artery dilation were shifted to 17- to 20-fold higher doses.

Hiremath et al. (1989) investigated the effect of systemically administered GTN (transdermal patches, ointment, and sublingual tablets) on α-adrenoceptor agonist-mediated constriction of human dorsal hand veins. GTN patches and ointment (15 to 60 mg/24 h) applied for 1 to 4 h did not modify the sensitivity (ED_{50}) to the α-adrenergic agonist phenylephrine in arteries. However, sublingual GTN (0.15 to 0.60 mg) administration caused significant relaxation of partially constricted veins. Following 24-h exposure to a GTN patch (15 mg/24 h), GTN dose-response curves were not altered, suggesting that there was no development of tolerance to transdermal GTN. The authors concluded that tolerance to transdermal GTN does not occur in veins.

D. Mechanism of Tolerance to Organic Nitrate Esters

Chronic exposure of tissues to drugs and hormones may lead to a reduction in the ability to respond to a new challenge by the same agonist, and the term "refractoriness" or "desensitisation" has been adopted to describe this phenomenon. Similarly, a reduced input of stimulatory influences may result in hypersensitivity to a particular hormone or drug (e.g., denervation hypersensitivity). Teleologically, such compensatory responses might be viewed as protective mechanisms that allow tissues to adapt to changes in the hormonal environment. Hormone-induced changes in target cell responsiveness appear to be a common event among different adenylate cyclase-coupled systems, as well as in many hormone-sensitive systems not involving adenylate cyclase (Tell et al., 1978; Davies and Lefkowitz, 1981). The most detailed knowledge currently available regarding the mechanisms behind desensitisation stems from work on β-adrenoceptors. Desensitisation of β-adrenoceptors appears to involve several processes, including uncoupling of the receptor from intracellular effector systems (e.g., G-proteins and adenylate cyclase), receptor sequestration, and receptor downregulation (i.e., lowering of the total number of receptors in the cell). These different processes apparently operate in different time frames and some involve phosphorylation events (Lefkowitz et al., 1990).

Tolerance to organic nitrate esters has been recognised as a pharmacological phenomenon for more than 100 years. The first description of tolerance to organic nitrate esters was by Stewart (1888), who reported tolerance to GTN in humans, and later several reports showed the existence of tolerance in humans and in experimental animals in vivo (Ebright, 1914; Crandall et al., 1931; Myers and Austin, 1929; Bogaert and de Schaepdryver, 1968; Bogaert, 1968; Clark and Litchfield, 1969). These findings prompted the investigation of the possible existence of tolerance in isolated vascular smooth muscle taken from animals made tolerant to organic nitrate esters in vivo (Needleman, 1970; Herman and Bogaert, 1971). Methods for inducing tolerance to organic nitrate esters in vitro were also devised (Needleman and Johnson, 1973; Needleman et al., 1973). As with other drugs, tolerance to organic nitrate esters can theoretically be explained by either pharmacokinetic factors and/or pharmacodynamic factors. Pharmacokinetic tolerance is usually considered to involve increased biodegradation and/or elimination of a drug, leading to a reduced plasma concentration of active drug. The persistence of tolerance in vascular smooth muscle prepared from animals made tolerant in vivo and the fact that tolerance can be readily induced in vascular smooth muscle in vitro suggest that pharmacokinetic factors may be less important than pharmacodynamic factors. However, in the case of organic nitrate esters, a biotransformation step at the level of target tissues is apparently of crucial importance for the action of these drugs. Therefore, two different pharmacokinetic events must be considered: one involved in degradation and/or elimination of the drug from the body and one involved in biotransformation of the organic nitrate ester at the level of vascular smooth muscle and other target tissues.

Currently no evidence exists demonstrating increased biotransformation or elimination during tolerance to organic nitrate esters (Clark and Litchfield, 1969; Needleman and Harkey, 1971; Needleman et al., 1971). Furthermore, evisceration of rats, which substantially reduces in vivo degradation of GTN, did not restore the blood pressure response to GTN in tolerant animals (Lang et al., 1972). Regarding the effect of tolerance on the target tissue biotransformation of organic nitrate esters, it was found that in vitro tolerance to GTN resulted in a significantly reduced level of dinitrates in the aortic wall, suggesting a reduced biotransformation of GTN in the vascular wall (Brien et al., 1986). Reduced levels of metabolites of both GTN and ISDN were found in tolerant rabbit aortic strips, and a significant cross-tolerance between GTN and ISDN regarding the effect on metabolite concentration within the vascular wall has also been reported (Slack et al., 1989). In addition, it has been shown that the preferential formation of the IS-5-MN metabolite and 1,2-GDN seen in nontolerant vascular tissue is markedly attenuated in tolerant tissue (Brien et al., 1988; Slack et al., 1989). Reduced biotransformation of organic nitrate esters and a loss of selective metabolite formation after tolerance induction have also been shown in various cultured cell lines of both vascular and nonvascular origin (Bennett et al., 1989). Thus far, there are essentially no studies concerning the effect of tolerance on the target tissue biotransformation of organic nitrate esters other than GTN and ISDN.

A question with some relevance to target tissue biotransformation is whether tolerance induces structural or functional changes in the vascular wall leading to altered tissue distribution of the organic nitrate ester or its metabolites. Sutton and Fung (1983) reported that the in vitro incorporation of radiolabeled GTN in portal vein from tolerant rats was significantly reduced compared with nontolerant animals, whereas tolerance had no effect on GTN uptake in aorta. However, in other studies no evidence has been obtained for an effect of tolerance on tissue uptake of the organic nitrate ester (Brien et al., 1986; Slack et al., 1989). Recently, we showed that aortas from GTN-treated rats contains higher levels of intact GTN than do aortas treated with a single dose of GTN; nonetheless, a marked tolerance to GTN can be shown in aortas from GTN-treated animals (Torfgård et al., 1990a). These results suggest that the absolute level of GTN in the target tissue is not a major determinant for the pharmacological effect of this nitrate ester.

The mechanism behind the reduced biotransformation

of organic nitrate esters in tolerant tissues is not entirely clear. One possible explanation could be a metabolite-induced inhibition of the enzyme systems involved, and some evidence for such an effect has been presented (Cossum and Roberts, 1985; Cossum et al., 1986). Metabolite inhibition may also be operative in degradation/elimination reactions and could possibly explain the reduced plasma clearance and accumulation of metabolites after infusion of organic nitrate esters in vivo, as shown for both GTN and ISDN (Sutton and Fung, 1984; Noonan et al., 1985; Chong and Fung, 1989). Another explanation could be that tolerance is due to a shortage of some essential cofactor that is necessary for target tissue biotransformation of organic nitrate esters and formation of guanylate cyclase-activating intermediates (such as nitric oxide or S-nitrosothiols). The pathways responsible for the biotransformation of organic nitrate esters have not been extensively characterised, and the enzymes involved are not unequivocally identified, although some evidence exists that glutathione S-transferase may be involved (see IV.A). There is also satisfactory evidence that one important factor explaining tolerance to organic nitrate esters is a reduction in critical SH-containing compounds acting as cofactors in target tissue biotransformation. This concept was first advanced by Needleman and Johnson (1973, 1976), who showed that tolerance to GTN in rat aorta was accompanied by a net loss of titrable tissue SH groups and that tolerance to GTN can be reversed by SH-reducing agents. Gruetter and Lemke (1985) found that the tissue content of cysteine and glutathione was also decreased in tolerant bovine coronary artery. However, these authors could not restore responsiveness by incubating tolerant vessels with cysteine or glutathione, despite the fact that tissue levels of thiols were significantly elevated by this procedure. Similarly, Abdollah et al. (1987) were unable to show an effect of N-acetylcysteine on in vitro GTN tolerance.

In contrast to these findings several authors have found that glutathione and N-acetylcysteine are able to prevent or reverse vascular smooth muscle tolerance to organic nitrate esters induced either in vitro or in vivo (Torresi et al., 1985; Berkenboom et al., 1988; Fung et al., 1988). There are several possible modes of action by which exogenously applied SH agents could restore target tissue responsiveness. One possible function of the SH compound may be to replete or regenerate intracellular thiols essential for target tissue biotransformation of organic nitrates. Another possible function could be to promote extracellular formation of reactive intermediates (S-nitrosothiols) necessary for activation of guanylate cyclase in the target tissue (Fung et al., 1988). In the latter case, the product formed must be able to penetrate the plasma membrane of the cells in the target tissue.

Several studies have shown that vascular smooth muscle tolerance to organic nitrate esters, induced either in vitro or in vivo, is accompanied by a reduced cGMP response; this has been demonstrated for various types of smooth muscle (peripheral arteries, coronary arteries and veins) of different species origin, including humans (Wikberg et al., 1980; Axelsson et al., 1982, 1985; Axelsson and Andersson, 1983; Keith et al., 1982; Axelsson, 1984; Ahlner et al., 1986b, 1987a; Rapoport et al., 1987). This tolerance could be seen even after incubation of isolated vascular smooth muscle with GTN concentrations as low as 0.1 nM for 2 h (Ahlner et al., 1987b), i.e., at concentrations of GTN comparable to those obtained in plasma in humans. Furthermore, it was found that the guanylate cyclase activity in cell-free preparations of tolerant vascular smooth muscle was decreased (Axelsson and Andersson, 1983; Axelsson and Karlsson, 1984; Waldman et al., 1986b; Mülsch et al., 1988a, 1989b) and that guanylate cyclase partially purified from GTN-tolerant rat aortas exhibited persistent desensitisation (Waldman et al., 1986b).

A similar marked reduction in guanylate cyclase activity was also found in cultured fibroblasts. In these cells the time-dependent recovery from GTN tolerance was partially prevented by the protein synthesis inhibitor cycloheximide (Schröder et al., 1988). This was interpreted to suggest that tolerance to GTN induced a stable modification of guanylate cyclase and that reversal of tolerance required de novo synthesis of enzyme. An effect of tolerance on the level of guanylate cyclase is also indicated by the finding that preincubation of coronary artery supernatant with GTN results in a markedly diminished guanylate cyclase activity (Romanin and Kukovetz, 1989). In this latter study, it was found that other organic nitrate esters, including ISDN and its metabolites, affected guanylate cyclase activity to a much lesser degree. It could be argued that the reduced cGMP response and the lower guanylate cyclase activity in tolerant tissue can be explained exclusively by a deficiency in critical SH groups necessary for biotransformation of organic nitrate esters. However, there is also a marked reduction in guanylate cyclase stimulation caused by nitricoxide as well as by compounds such as SNP and 3-morpholinosydonimine, considered to release nitric oxide directly without the requirement of a preceding metabolic step (Axelsson and Andersson, 1983; Axelsson and Karlsson, 1984; Waldman et al., 1986b; Schröder et al., 1988; Mülsch et al., 1988a, 1989c; Romanin and Kukovetz, 1989). Furthermore, addition of various SH agents does not fully restore guanylate cyclase activation induced either by organic nitrate esters or by more directly acting guanylate cyclase activators, such as SNP or 3-morpholinosydonimine (Kukovetz and Holzmann, 1985; Axelsson and Karlsson, 1984; Romanin and Kukovetz, 1989). This seems to support the assumption that tolerance to organic nitrate esters is at least partly explained by a direct effect on guanylate cyclase.

However, in contrast to these reports, other authors have found tolerance to organic nitrate esters to be quite selective, with minimal cross-tolerance to the more directly operating guanylate cyclase activators (Kukovetz and Holzmann, 1986; Berkenboom et al., 1988; Mülsch et al., 1989b; Henry et al., 1990; Bauer and Fung, 1991). The reason for these discrepancies is not clear, although the dose of drug used for tolerance induction is probably of importance, and species differences may exist, as well as differences between various vascular regions. It should be noted that, to a certain degree, there is also development of tolerance to SNP and 3-morpholinosydonimine and cross-tolerance between these compounds and organic nitrate esters (Axelsson et al., 1982; Henry et al., 1989). In two studies by Henry et al. (1989, 1990), it was found that bovine coronary arteries made tolerant to GTN in vitro were also tolerant to ISDN. However, tolerance to ISDN and GTN showed different characteristics; there was a reduction in the maximal response to GTN, whereas the concentration-effect curves for ISDN were shifted to the right without any reduction in the maximal relaxation. This difference was most marked at medium-low dose tolerance and could indicate that the mechanism of action and the mechanisms behind tolerance induction are not identical for different organic nitrate esters. Similar findings with a reduction in both maximal relaxation and potency of GTN were found in rat aorta isolated from rats made tolerant to GTN in vivo (Torfgård et al., 1990a).

There are several possible ways in which guanylate cyclase, at least theoretically, could be affected by tolerance induction: (*a*) modification of the enzyme molecule could be brought about by oxidation of SH groups necessary for expression of full enzymatic activity; (*b*) modification of the heme iron ($Fe^{2+} \rightarrow Fe^{3+}$) could occur, rendering the enzyme nonresponsive to nitric oxide; (*c*) the enzyme molecule could be modified by phosphorylation, either via cG-Pk, which would thus mediate a negative feedback signal, or via some other protein kinase; and (*d*) the amount of enzyme could be reduced, possibly as a result of decreased transcription or translation of mRNA for guanylate cyclase. The first two alternatives seem entirely possible. This is true in view of the key role known to be played by SH-containing compounds in the biotransformation of organic nitrate esters, with simultaneous oxidation of SH groups, and the ability of organic nitrate esters to oxidise hemoglobin to methemoglobin (see preceding section). The third and fourth alternatives are purely speculative. Phosphorylation of rat brain-soluble guanylate cyclase by cAMP-dependent protein kinase has been reported, although this phosphorylation was found to increase enzyme activity (Zwiller et al., 1981b); phosphorylation of particulate guanylate cyclase was likewise found to increase enzyme activity (Ramarao and Garbers, 1988).

In vitro determination of phosphorylation of purified guanylate cyclase by cG-Pk, and possibly other protein kinases, may give a hint as to whether this mechanism can be of importance in explaining tolerance. Because antibodies directed against soluble guanylate cyclase have been produced, and because soluble guanylate cyclase has been cloned, it should, in the near future, also be possible to investigate the importance of a transcriptional and/or translational regulation of guanylate cyclase during tolerance.

In addition to a reduction in guanylate cyclase activity as a result of tolerance development, it has also been shown that the phosphodiesterase activity is increased in aortas from GTN-tolerant rats (Axelsson and Andersson, 1983). The effect on the phosphodiesterase activity was absent or much less pronounced after in vitro tolerance induction (Axelsson and Karlsson, 1984; Ahlner et al., 1986b), indicating that the effect on this activity develops more slowly than the effect on guanylate cyclase. The exact mechanism behind the increased phosphodiesterase activity, however, remains to be elucidated.

Tolerance to organic nitrate esters does not seem to impair the function of formed cGMP and apparently does not affect particulate guanylate cyclase because the relaxation and cGMP increase induced by 8-Br-cGMP and ANP are not significantly affected by tolerance development (Keith et al., 1982; Molina et al., 1987; Rapoport et al., 1987; Schröder et al., 1988; Ekstam Ljusegren et al., 1988). Possible cross-tolerance between organic nitrate esters and endothelium-dependent relaxations has been studied, and contradictory reports have appeared; some authors have found a marked cross-tolerance between GTN and EDRF, with regard to both relaxation and cGMP increase (Molina et al., 1987; Rapoport et al., 1987; Ekstam Ljusegren et al., 1988), whereas others could not demonstrate such an effect (Mülsch et al., 1988a, 1989b; Van der Vorde et al., 1987; Kowaluk and Fung, 1988; Henry et al., 1989).

The reason for these discrepancies is not clear, although a partial explanation could be due to differences in experimental design. Thus, GTN tolerance may reduce the release of EDRF, which would affect the response to EDRF-releasing agents in intact vascular specimens. In contrast, no effect would be seen in bioassay experiments using cultivated endothelial cells as EDRF donors and endothelium-denuded vascular preparations for tolerance induction and measurement of relaxation. There are indications that different endothelium-derived relaxing substances exist and that nitric oxide released from the endothelium may induce smooth muscle relaxation through at least two different mechanisms (i.e., cGMP elevation and hyperpolarisation). These mechanisms may be affected differently by tolerance induction, depending on the type of vascular smooth muscle investigated and the concentration and type of organic nitrate ester used. Furthermore, to firmly establish the occurrence of cross-tolerance between organic nitrate esters

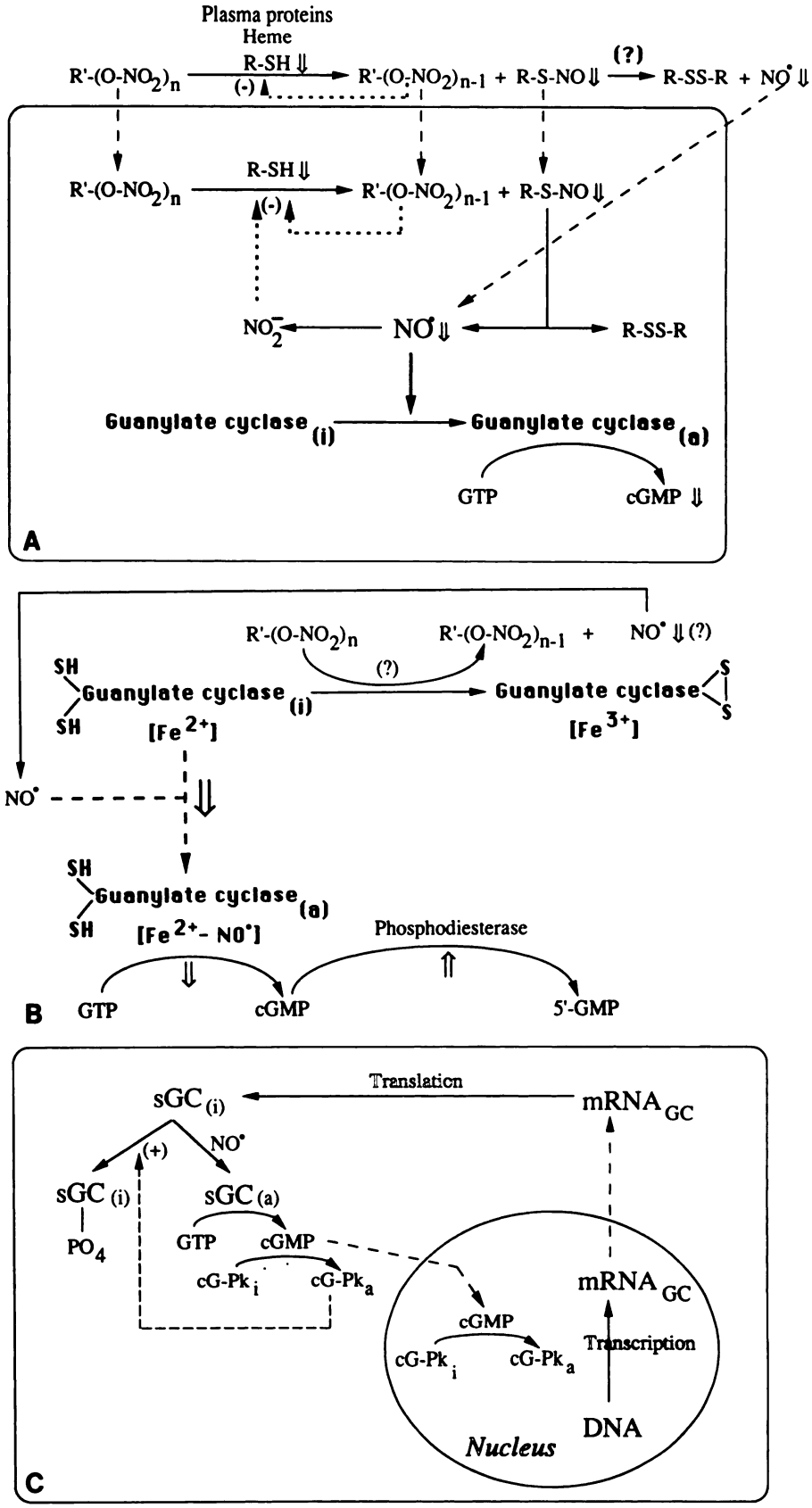

FIG. 8. Schematic drawing showing different mechanisms that may explain tolerance to organic nitrate esters. To simplify the presentation, the different mechanisms are shown separated from each other, although they are not to be regarded as mutually exclusive. A, Putative mechanism for tolerance development involving changes in target tissue conversion of organic nitrate esters to nitric oxide. The formation of nitric oxide is reduced through a depletion (⇓) of critical tissue or plasma thiol compounds (R-SH). The metabolising enzymes may also be

and endothelium-derived relaxing mechanisms, it is desirable to measure the relaxation induced by EDRF-releasing agents throughout an extended concentration range, both in control and tolerant tissues. Some of the possible mechanisms explaining tolerance to organic nitrate esters on a cellular level are presented in fig. 8.

In addition to a direct effect on the target tissue, activation of compensatory reflexes has also been suggested as a mechanism to explain tolerance to organic nitrate esters (Clark and Litchfield, 1969; Rush et al., 1971; Imhof et al., 1989). Activation of such biological counterregulatory mechanisms is sometimes referred to as pseudotolerance (Stewart et al., 1986), and seems to be of some importance in in vivo conditions but can obviously not explain tolerance on the level of isolated vascular smooth muscle. In the in vivo study by Stewart et al. (1986) in dogs, it was shown that long-term exposure to GTN results in tolerance to its venodilating effects, whereas the arteriolar dilation is impaired by compensatory reflexes.

In patients with congestive heart failure, there is some evidence that reflex neurohumoral activation and a capillary fluid shift from the extravascular to intravascular space might be involved (Dupuis et al., 1990a,b; Packer et al. 1987). Packer et al. (1987) showed that the attenuation of the hemodynamic effects of an i.v. infusion of GTN in patients with congestive heart failure was accompanied by an increase in heart rate, plasma renin activity, and body weight, whereas another study in which a a large transdermal dose (90 mg/24 h) was given to patients with chronic congestive heart failure did not show any changes in serum catecholamines and renin concentrations as hemodynamic attenuation developed (Elkayam et al., 1985). Recently, two studies of tolerance to i.v. GTN in patients with congestive heart failure showed an increase in intravascular volume and plasma renin activity and a decrease in plasma ANP levels (Dupuis et al., 1990a,b). This indicates that multiple mechanisms might interact to produce development of tolerance to GTN in the clinical setting (Packer, 1990).

E. Effect of Sulfhydryl Replenishment on Tolerance

As mentioned before, a widely accepted theory for the mechanism of tolerance implies that during continued nitrate exposure there is a deficiency of reduced SH groups in vascular smooth muscle (Needleman et al., 1973; Ignarro et al., 1981b). Studies have suggested that the administration of reduced SH groups may potentiate the effect of GTN, diminish tolerance development, or even reverse tolerance. However, both in vitro and in vivo studies have yielded conflicting results.

Torresi et al. (1985) investigated the effect of N-acetylcysteine in the prevention of nitrate tolerance in incubated bovine coronary artery rings. The degree of tolerance that developed during nitrate administration was markedly reduced by administration of N-acetylcysteine. However, in other studies performed in vitro, it has not been possible to show any reversal of tolerance (Gruetter and Lemke, 1986; Abdollah et al., 1987).

Münzel et al. (1989) studied the effect of N-acetylcysteine on nitrate responsiveness of epicardial arteries and the venous system in dogs during long-term GTN treatment (1.5 μg/kg/min i.v. for 5 to 6 days). In dogs with GTN-specific tolerance (shift of venous or epicardial artery dilation to 15- to 17-fold higher dosages), N-acetylcysteine (100 mg/kg i.v.) had no dilator effect and did not alter the dose-response relationship for GTN. However, in nontolerant dogs, N-acetylcysteine augmented (1.5- to 2-fold) the dilation of epicardial arteries and the reduction of peripheral vascular resistance induced by 0.5 to 1.5 μg/kg/min GTN. In vitro, the stimulation of purified guanylate cyclase activity by GTN (10 to 100 μM) was potentiated by N-acetylcysteine (0.01 to 1.0 mM) in saline or in canine plasma, but N-acetylcysteine alone was ineffective.

In a recent study of rats, Newman et al. (1990) found that N-acetyl-L-cysteine prevented the development of tolerance to GTN, whereas N-acetyl-D-cysteine did not. The stereospecificity in the effect of N-acetylcysteine to prevent specific tolerance to GTN suggests that the interaction between GTN and N-acetylcysteine and/or cysteine involves an enzyme-dependent step. It has also been shown that the angiotension-converting enzyme inhibitor captopril may prevent or reverse GTN tolerance in vitro, probably due to the presence of a reactive SH group in the captopril molecule which would, thus, function in a similar way as N-acetyl-L-cysteine (Lawson et al., 1991).

Horowitz et al. (1983) carried out a study in humans in which the effects of GTN on mean arterial blood pressure and PCWP were recorded; this was followed by infusion of N-acetylcysteine. The procedure resulted in a significant decrease in the dose of GTN required to lower systemic arterial pressure and left ventricular filling pressures. In another investigation, Winniford et al.

subject to product inhibition (−) from nitrite or denitrated organic nitrate ester residues ($R'\text{-}(O\text{-}NO_2)_{n-1}$). This would result in a reduction (↓) in cGMP formation. B, Tolerance to organic nitrate esters may be due to a reduced (↓) guanylate cyclase activity and/or increased (↑) phosphodiesterase (PDE) activity. It is suggested that prolonged exposure to organic nitrate esters in high concentrations may result in a modification of guanylate cyclase, either due to oxidation of critical SH groups located on the enzyme or an oxidation of the heme iron. Both of these changes may result in a reduced enzyme activity and refractoriness to stimulation by nitric oxide. C, Hypothetical mechanisms for tolerance development involving changes in transcription of the gene(s) for soluble guanylate cyclase and/or translation of mRNA coding for soluble guanylate cyclase. A putative mechanism for tolerance development due to phosphorylation of soluble guanylate cyclase, with enzyme inhibition as a result, is also shown. This latter mechanism may be regarded as a direct negative feedback loop, provided that cG-Pk is the mediator of the phosphorylation event. i, inactive enzyme; a, active enzyme.

(1986) examined the effect of GTN on coronary sinus blood flow in 18 patients. The coronary sinus blood flow was measured by thermodilution before and during intracoronary administration of GTN (25 μg) both before and 5 min after a 15-min i.v. infusion of 5% dextrose in water or 100 mg/kg N-acetylcysteine. After N-acetylcysteine, coronary sinus blood flow increased markedly with GTN ($P < 0.01$) compared to after the injection of GTN alone. In both studies it was suggested that N-acetylcysteine, a source of reduced SH groups, potentiates the vasodilating effect of GTN.

May et al. (1987) studied 19 subjects (17 with coronary artery disease). Coronary sinus blood flow, which approximates blood flow to the left ventricle, was measured before and during intracoronary injections of GTN (10, 25, 50, and 100 μg). The patients then received a 24-h i.v. infusion of saline or of GTN (45 ± 13 μg/min, mean ± SD), after which the responses of coronary sinus flow to the same doses of intracoronary GTN used earlier were measured. In the patients given saline, GTN caused similar percentage increases in coronary sinus flow before and after the saline infusion. In the 12 patients given i.v. GTN, the four intracoronary doses caused significantly smaller percentage increases in coronary sinus flow, indicating the development of partial tolerance. Subsequently, seven of the 12 last-mentioned patients received N-acetylcysteine, after which intracoronary GTN caused percentage increases in coronary sinus flow similar to the values measured before the i.v. GTN was given. The authors concluded that the coronary vasodilator effect of GTN is attenuated by an i.v. infusion of GTN and that tolerance to the agent can be reversed by administration of the SH-group donor N-acetylcysteine. Similar results were also obtained after treatment of GTN-tolerant human volunteers with methionine, an amino acid capable of replenishing SH groups (Levy et al., 1991).

Horowitz et al. (1988) compared the effects of i.v. GTN combined with i.v. N-acetylcysteine (5 g every 6 h) to those of i.v. GTN given alone; a double-blind trial was performed on 46 patients with severe unstable angina pectoris unresponsive to conventional treatment. Treatment with the combination and that with GTN alone was associated with a similar frequency of episodes of chest pain and of increments in GTN infusion rate for pain control. However, the GTN/N-acetylcysteine group had a significantly lower incidence of acute myocardial infarction than did the GTN/placebo group. Symptomatic hypotension occurred frequently in the combined therapy group. Lactate/pyruvate ratios and venous GTN concentrations were not significantly affected by N-acetylcysteine.

Packer et al. (1987) studied 35 patients with severe congestive heart failure. A high dose (6.4 μg/kg/min) i.v. infusion of GTN was given either continuously or intermittently (12-h infusions separated by intervals of 12 h). In all patients i.v. GTN produced immediate hemodynamic benefits, i.e., reduced systemic pressure and left ventricular filling pressures. The magnitude of this improvement was greatly diminished after 48 h of continuous therapy with the drug. This attenuation was accompanied by cross-tolerance to oral ISDN and by increases in heart rate, plasma renin activity, and body weight. In contrast, intermittent therapy with i.v. GTN was not associated with a loss of hemodynamic efficacy or cross-tolerance to oral nitrates and was not accompanied by changes in neurohormonal activity or body weight. In eight patients in whom nitrate tolerance developed during continuous i.v. therapy, the administration of the SH-containing compound N-acetylcysteine (200 mg/kg orally) restored the hemodynamic state to that observed at the start of the infusion of GTN (i.e., partial reversal of tolerance). The authors concluded that neurohormonal activation and depletion of SH groups may interact to cause the loss of hemodynamic efficacy that occurs during prolonged treatment with i.v. GTN in patients with heart failure.

In a study by Hastrup Svendsen et al. (1989) it was shown that a large oral dose (2,400 mg × 2) of N-acetylcysteine given together with a single oral dose of IS-5-MN (60 mg) significantly prolonged the total exercise time as compared with treatment with placebo and IS-5-MN. Other studies have been unable to show any effect of N-acetylcysteine on the development of tolerance to nitrates. In a study by Hogan et al. (1989b), seven volunteers were treated in a double-blind randomised crossover manner for two 4-day periods with 20 mg of transdermal GTN/24 h together with N-acetylcysteine (200 mg three times daily) or matching placebo. Hemodynamic measurements (blood pressure and heart rate) at rest and following maximal treadmill exercise were performed before treatment and 4 h after starting treatment on days 1 and 4. Significant hemodynamic changes as evidenced by a decrease in blood pressure and increase in heart rate, were seen on day 1 in both the N-acetylcysteine and placebo phases. By day 4 the hemodynamic changes had returned to the pretreatment values during both drug and placebo phases, suggesting the development of tolerance in both treatment groups.

Parker et al. (1987b) assessed the hemodynamic and antianginal effects of 30 mg ISDN on 12 patients with chronic stable angina, both after initial dosing and after 7 to 10 days of therapy four times daily. During early therapy, ISDN produced significant hemodynamic and antianginal effects that persisted during a 3-h observation period. During sustained therapy, there was an attenuation of the hemodynamic effects at rest, and treadmill exercise time to the onset of angina and to the development of moderate angina was increased only 1 h after dosing; no effect was apparent at 3 h. During this state of nitrate tolerance, patients were treated with an infusion of normal saline or 100 mg/kg N-acetylcysteine,

and exercise testing was repeated. N-acetylcysteine did not change the hemodynamic findings at rest or during exercise, and there was no improvement in exercise tolerance.

It has not been determined whether tolerance development occurs to the inhibitory effects of nitrates on platelet aggregation. Regarding the effect of N-acetylcysteine, Stamler et al. (1988) found that N-acetylcysteine alone, in a dose-dependent manner, inhibited ADP-induced platelet aggregation in vitro and that this compound when added ex vivo to platelets from patients treated with GTN i.v. had a more pronounced effect than when added to platelets from patients not treated with GTN.

In conclusion, some patients seem prone to develop tolerance to the action of the nitrates, whereas, at the other extreme, some patients do not develop clinically important tolerance at all. The reason for such interindividual differences is not known, and at present there is no available method that allows recognition in advance of patients with a high risk for tolerance development. To minimise the risk for most patients, it is essential to select a dosage regimen that is optimal. Such regimens should include some kind of so-called nitrate-free interval. It is not possible to state how long the interval should be, but 10 to 12 h might be sufficient. Finding the right dosage regimen is the only practical way to avoid the development of tolerance; the use of a reduced SH group donor such as N-acetylcysteine is not a suitable approach for clinical practice because, in the studies cited above, very high doses were given and the results are contradictory.

F. Nitrate Dependency

Dependence on a drug means that psychic and/or physical withdrawal symptoms will appear when the substance is discontinued. Withdrawal syndromes are typical of various mood- and behaviour-altering drugs, including alcohol. Frequently, a rebound effect occurs with apparent increased susceptibility in physiological systems affected by the compound. Although tolerance and physical dependence commonly occur together, one may exist without the other.

Many reports have documented characteristic withdrawal symptoms in munitions workers. These consist of typical severe headaches in exposed people who are absent from the nitrate-laden environment for 2 to 3 days (Laws, 1910; Ebright, 1914). To avoid the headaches, the employees rubbed GTN into their skin or wore impregnated headbands on weekends or holidays. In addition to the headaches, more serious sequelae to nitrate withdrawal have been reported. It is well-known that GTN-dependent individuals may experience symptoms of angina pectoris, usually within 48 to 72 h after withdrawal from exposure (Hogstedt and Andersson, 1979; Hogstedt, 1980; Lange et al., 1972). Direct angiographic evidence for spasm in normal coronary arteries has been obtained from subjects experiencing withdrawal symptoms (Lange et al., 1972; Klock, 1975). The prevalence of withdrawal symptoms among individuals exposed to organic nitrate esters has been estimated to be about 5% (Lange et al., 1972). There are also indications that the risk of sudden death is enhanced among explosives workers, especially after withdrawal from nitrate exposure (Carmichael and Lieben, 1963; Lund et al., 1968; Lange et al., 1972; Klock, 1975; for review see also Morton, 1977).

1. A clinical problem? The safety of abrupt withdrawal of nitrates from patients with angina pectoris has been discussed and some experts have recommended that nitrates should not be withdrawn abruptly (Abrams, 1980). However, no systematic study of nitrate withdrawal in patients has been published. Reeves et al. (1985) addressed the question in an animal model. They found that rabbits treated with GTN for 6 weeks before withdrawal of the drug developed myocardial ischemia, ventricular arrhythmias, and myocardial infarctions when exposed to ergonovine and indomethacin. A control group did not show any ECG changes when challenged with ergonovine and indomethacin.

Several anecdotal reports indicate intensification of angina and even sudden death after withdrawal of nitrates. Franciosa et al. (1978) described two patients with coronary artery disease who died suddenly after discontinuing oral ISDN. Muller and Gunther (1978) described a case of acute myocardial infarction occurring soon after withdrawal of GTN in a patient with variant angina.

In a single-blind study of 32 patients with angina pectoris, Rehnqvist et al. (1988) examined the effect of abrupt withdrawal of controlled-release IS-5-MN after at least 1 year of drug administration. After 2 weeks of placebo treatment, IS-5-MN was reinstituted, and the patients were observed for another 2 weeks. After discontinuing the drug, three of the patients experienced severe anginal symptoms necessitating hospitalisation. Patients complained of more severe anginal symptoms during the placebo period and also experienced more frequent anginal attacks and used more GTN tablets than during active treatment. ST-segment changes during exercise were pronounced with placebo; after the drug was reintroduced, these variables improved significantly. On the other hand, no deterioration occurred in exercise performance during the placebo phase. The authors concluded that abrupt withdrawal should not be recommended because of the possibility of severe exacerbation of anginal symptoms, but they also remarked that no clear-cut rebound phenomena were seen.

Ferratini et al. (1989) in a double-blind crossover study (two 15-day periods) compared continuous and intermittent (12-h nitrate-free interval) application of 20 mg GTN/24 h. During the 15-day period, nighttime withdrawal of GTN patches resulted in a total of 11 nocturnal anginal attacks in six of 10 patients, whereas no anginal

attacks at night were registered during 24-h application of the patch or before or during wash-out. Cowan et al. (1987a) reported that there was no evidence for exacerbation of anginal symptoms in the intermittent phase during overnight placebo treatment in comparison with the continuous active treatment group. This is in agreement with the study of Schaer et al. (1988) in which overnight rebound was not observed in patients according to histories or ambulatory Holter monitoring calibrated for ST-segment analysis.

In a study by DeMots et al. (1989), it was noted that nine patients in the intermittent treatment group experienced an increase in nonexertional angina during patch-off periods, and in this multicentre study one death was reported during the patch-free period of active therapy. In patients with congestive heart failure, Packer et al. (1986) found rebound increases in mean arterial pressure and systemic vascular resistance and decreases in cardiac index after removal of transdermal GTN (10 to 60 mg/24 h). The authors speculated that this was due to an activation of the renin-angiotensin system.

The hemodynamic and hormonal responses to GTN administered transdermally (patch) were evaluated in nine patients with severe congestive heart failure and in nine normal subjects (Olivari et al., 1983). In patients with heart failure GTN produced sustained hemodynamic effects that fully persisted for at least 6 h. A significant decrease in right and left ventricular filling pressures was associated with an increase in stroke index and a significant decrease in forearm and pulmonary vascular resistance. There was no change in heart rate and systemic arterial pressure or in plasma norepinephrine or plasma renin activity. After 24 h, pressures had partially returned to control levels, but mean pulmonary artery pressure was still significantly lower than in the control period. After removal of GTN, each patient exhibited a decrease in cardiac index and an increase above control values in pulmonary and systemic arterial pressures and pulmonary, systemic, and forearm vascular resistance. This transient rebound appeared to be unrelated to stimulation of the sympathetic or renin-angiotensin system.

Further studies are required to evaluate the clinical importance of possible nitrate dependency. Firm knowledge regarding this issue is important when recommending interval therapy with nitrate-free intervals.

2. Mechanism of nitrate dependency. Several investigators have attempted to simulate the human exposure situation in test animals. Yoshikawa (1965) found that prolonged treatment of mice with EGDN resulted in an increased sensitivity to adrenaline. At an early stage of the EGDN treatment, the rate of mortality caused by adrenaline increased temporarily, whereafter it decreased gradually to control values during continued EGDN exposure. During the withdrawal period, there was again an abrupt increase in adrenaline sensitivity.

Withdrawal from GTN exposure also increases adrenaline sensitivity in mice (Rydell and Axelsson, 1984); this was found to be due to an increased α_1-adrenoceptor sensitivity. Vigliani et al. (1968) reported an increased sensitivity to the cardiotoxic effects of tyramine and amphetamine, combined with a reduced exercise tolerance in EGDN-treated mice and rats. These effects coincided with an elevated catecholamine content in the hearts of these animals, and this finding was suggested to explain the supersensitivity to the sympathomimetic drugs. In a later study by other investigators, evidence was found for a supersensitivity to adrenaline-induced arrhythmias and blood pressure elevation in EGDN-exposed rats (Clark, 1970). The most marked supersensitivity occurred 24 h after the last injection of EGDN, and this was preceded by a period of subsensitivity. It was suggested that the nervous system was involved in the supersensitisation, because the increased adrenaline sensitivity was absent in pithed rats. The supersensitivity was proposed to be due to a temporary deficiency in circulatory-compensating mechanisms.

Prolonged EGDN treatment has also been shown to lower catecholamine turnover in rat brain (Johansson et al., 1987). Further evidence for a pronounced dependency has also been obtained in rabbits, in which abrupt withdrawal of GTN treatment resulted in marked ECG changes, ventricular tachycardia, and myocardial infarctions (Reeves et al., 1985). Results from animal studies such as those described above have lead to the speculation that the increased incidence of angina pectoris and sudden death among workers exposed to GTN and EGDN might be due to an increased sympathetic activity (Hogstedt, 1980). The exact mechanism explaining the augmented sensitivity to sympathetic stimuli during the withdrawal period is not known. One possible factor could be excessive vasoconstriction; this assumption is supported by the discovery that α_1-adrenoceptor blockers provided specific protection against the lethal action of adrenaline, whereas administration of β-adrenoceptor antagonists potentiated its toxic action (Rydell and Axelsson, 1984). This could be due to either a sensitisation of α_1-adrenoceptors (mediating vasoconstriction) or to a desensitisation of β-adrenoceptors (mediating vasodilation). An increased in vitro sensitivity to contractile stimuli has also been shown in aortas isolated from GTN- and EGDN-pretreated rats (Axelsson and Andersson, 1982; Johansson et al., 1987).

Another possible mechanism is that the withdrawal symptoms are caused by alterations distal to the adrenoceptor, as, for example, the reduced effectiveness of the cGMP system (mediating vasodilation) (Axelsson et al., 1982; Axelsson and Andersson, 1983). It has previously been suggested that cGMP may function as a negative feedback signal which counteracts the increase in intracellular calcium concentration that occurs during smooth muscle contraction (Schultz et al., 1973, 1975;

Andersson et al., 1980). In this context, it is interesting to note that several cell types, including platelets and vascular smooth muscle, possess a calcium-dependent pathway for the synthesis of nitric oxide, which can activate soluble guanylate cyclase (Lückhoff et al., 1988; Mülsch et al., 1989a; Busse and Mülsch, 1990; Radomski et al., 1990; Wood et al., 1990). It could be speculated that this pathway may function as a cGMP-mediated regulatory mechanism that works to counteract excessive increases in intracellular calcium concentrations. The effect of tolerance and organic nitrate ester dependency on this endogenous counterregulatory system remains to be investigated.

An interesting observation regarding organic nitrate ester dependency is that the withdrawal symptoms of dynamite workers are, in many aspects, similar to the symptoms of variant (Prinzmetal's) angina. Both of these conditions are characterised by the following symptoms: diffuse spasm of the large coronary arteries, characteristic changes in ST-segment and T waves in ECGs, and the experience of angina pectoris during rest, often in the early morning hours (Lange et al., 1972; Hillis and Braunwald, 1978). Furthermore, patients with variant angina also seem to exhibit increased sensitivity to coronary vasoconstriction induced by α-adrenoceptor stimulation (Curry et al., 1977; Hillis and Braunwald, 1978), and β-adrenoceptor-blocking agents may, in some instances, worsen these symptoms, which is in contrast to what is observed in patients with classical angina pectoris due to atherosclerotic lesions in the coronary arteries. These findings are in close agreement with the characteristics of adrenaline toxicity in GTN-tolerant mice (Rydell and Axelsson, 1984).

IX. Drug Interactions and Side Effects

A. Drug Interactions

Few interactions between organic nitrates and other drugs have been described, and very few seem to be of any clinical importance.

1. Aspirin and indomethacin. Weber and coworkers (1983) reported that pretreatment with 1 g of aspirin increased plasma levels of GTN by about 50%. In another study, 0.8 mg of GTN was given sublingually to healthy volunteers on three different occasions: once without other medication, once after 1 g aspirin and 2 h before GTN, and once after 8 days of treatment with aspirin (Rey et al., 1983). After both aspirin treatments, the plasma concentrations of GTN tended to be higher, but no statistically significant changes were reported. Aspirin was not found to have any effect on ISDN-induced coronary dilation in patients (Simonetti et al., 1983). Indomethacin, like aspirin, inhibits prostaglandin synthesis but did not affect the circulatory and antianginal effects of GTN in patients with chronic stable angina pectoris (Thadani and Kellerman, 1983).

Clinical importance of the interactions between inhibitors of prostaglandin synthesis and organic nitrates has not been confirmed.

2. Heparin. Habbab and Haft (1987) reported that intravenously administered GTN may interfere with the anticoagulant effect of heparin. Col et al. (1985) stated earlier that such an interaction could take place, although they suggested that it was the propylene glycol diluent in the GTN solution that had caused the effect. Habbab and Haft (1987) were able to show that the interaction took place whether propylene glycol was present or not. In a clinical study of 27 patients, Pizzulli and coworkers (1988) found that when GTN infusion was added to a heparin infusion the APTT decreased significantly from 130 ± 28 to 60 ± 23 s (mean ± SD; $P < 0.01$). Following termination of the GTN infusion, the APTT returned to the initial value (126 ± 30 s). In nine of the patients, heparin concentrations were also measured, and the concentration of heparin was unchanged whether GTN was infused simultaneously or not (Pizzulli et al., 1988). Thus, GTN seems to induce a reversible heparin resistance when infused simultaneously. The results were the same both when the solution did and when it did not contain propylene glycol. In contradiction to this observation, Lepor and coworkers (1989) could not find any influence of GTN on APTT when studying the effect of a short duration (1 to 2 h) infusion of GTN.

Becker et al. (1990) investigated the mechanism behind such an interaction. The patients studied ($n = 18$) were being treated in the critical care unit for either acute myocardial infarction or unstable angina. Using serial determinations, Becker et al. (1990) measured APTT, serum heparin concentration, antithrombin III antigen, and antithrombin III activity. The patients were divided into four treatment groups: (*a*) i.v. GTN and i.v. heparin, (*b*) i.v. GTN alone, (*c*) i.v. heparin alone, and (*d*) neither GTN nor heparin. Overall, APTT, heparin dose, heparin concentration, antithrombin III antigen, and antithrombin III activity did not differ significantly in patients receiving i.v. GTN compared with the other groups. However, patients receiving i.v. GTN at a rate exceeding 350 μg/min had a lower APTT ($P < 0.05$), a lower antithrombin III activity ($P = 0.02$), and a larger heparin dose requirement than patients receiving infusions at lower rates. The authors concluded that i.v. GTN-induced heparin resistance occurs at a critical GTN dose. A GTN-induced qualitative antithrombin III abnormality may be the underlying mechanism. However, Amin and Horrow (1990) did not see any effect of varying concentrations of GTN and heparin on antithrombin III concentration or activity studied in vitro.

The conclusion must be that patients treated with simultaneous i.v. GTN and i.v. heparin must be frequently monitored to avoid inadequate anticoagulation, and to avoid hemorrhage, heparin dosage should be decreased when stopping i.v. GTN therapy.

3. Vasodilators and other antianginal drugs. There is no true interaction between nitrates and other vasodilators or other antianginal drugs such as β-blockers. However, nitrates do potentiate the effects of all these agents. The problem of GTN-induced orthostatic hypotension may be more pronounced in patients concomitantly treated with other drugs that tend to cause hypotension. In patients treated with β-blockers, the increase in heart rate in response to GTN-induced vasodilation is inhibited, and orthostatic hypotension may develop (Hyldstrup et al., 1983).

4. N-acetylcysteine. It has been shown that the hemodynamic (and antianginal) effects of GTN might be enhanced by concomitant administration of N-acetylcysteine (Horowitz et al., 1983; Winniford et al., 1986). N-acetylcysteine in large oral doses (2,400 mg × 2) increased the exercise capacity in patients taking IS-5-MN (Hastrup Svendsen et al., 1989).

Horowitz et al. (1988) gave extremely large i.v. doses of N-acetylcysteine (20 g/day) together with i.v. GTN to patients with unstable angina; this treatment increased the risk of development of hypotension as compared with GTN infusion alone.

5. Captopril. Captopril, an angiotensin-converting enzyme inhibitor containing an SH moiety, has been reported to augment the circulatory effect of ISDN (van Gilst et al., 1987).

B. Side Effects

The nitrates are generally well tolerated and have few side effects. The adverse effects that do exist are mainly attributed to the vasodilatory effects of the drugs. Such side effects include headaches, hypotension, postural hypotension, impaired coronary and cerebral perfusion, dizziness, syncope, weakness, flushing, and palpitations. The occurrence of headaches is the most common adverse effect of the nitrates, appearing in many patients after initiation of treatment but usually disappearing with continued treatment; this side effect is the major cause of discontinuation of treatment.

A serious adverse effect resulting from the hemodynamic effects of the nitrates is a decrease in coronary perfusion pressure due to a decrease in systemic arterial pressure. This potential risk is minimised, especially in patients receiving i.v. nitrates, by appropriate monitoring. The most serious side effect is the combination of hypotension and bradycardia, which may be fatal. As early as 1887, the first report appeared of a patient exhibiting clinical shock associated with a very low and irregular pulse following GTN administration (Noer, 1887). This syndrome has been reported in patients taking sublingual GTN (Nemerovski and Shah, 1981; Come and Pitt, 1976) and in patients receiving i.v. GTN (Page et al., 1981; Come and Pitt, 1976; Flaherty et al., 1982). The incidence of this adverse effect has varied. Flaherty et al. (1982) reported an incidence of 4%, Page reported that two of 67 patients experienced this serious side effect; Come and Pitt reported seven episodes of hypotension and bradycardia in five patients receiving either sublingual (two) or i.v. (three) GTN. Four of the patients were hemodynamically monitored and showed reductions in left ventricular filling pressure simultaneously with the hypotension-bradycardia event. No initial increase in heart rate was observed in any of the patients before the appearance of bradycardia.

Combined hypotension and bradycardia has also been described in three of 18 normal subjects who had received 5 mg ISDN sublingually (Spörl-Radun et al., 1980).

Severe arterial hypotension usually develops suddenly, within 5 to 10 min after sublingual administration, and is accompanied by bradycardia. In nearly all reported cases, episodes could be rapidly reversed by increasing venous return (Trendelenburg position) and/or administrating atropine. A possible explanation for the hypotension-bradycardia syndrome is that the nitrate-induced reduction in venous return might result in a more pronounced diminution of an already low normal end-diastolic ventricular volume. The vigorous contractions of the relatively empty ventricles then leads to deformation of neural receptors, thereby eliciting vagal reflexes similar to the Bezold-Jarisch reflex (Thoren et al., 1976).

Nitrates can oxidise the iron in the hemoglobin molecule from the ferrous to the ferric state and form methemoglobin, a form that cannot transport oxygen. There is a risk that this might occur in patients receiving high doses of i.v. GTN (Kaplan et al., 1985). Gibson et al. (1982) reported that infusion rates higher than 7 μg/kg/min were associated with methemoglobinemia. This complication should be considered in any patient treated with GTN who appears cyanotic in the presence of normal arterial oxygen saturation. Methemoglobin levels higher than 3% indicate toxicity.

One report has claimed that GTN might further increase an elevated intracranial pressure (Rogers et al., 1979). No other reports confirm this, but giving nitrates to patients with elevated intracranial pressure is not recommended.

Regarding i.v. administration of GTN, there have been reports of adverse effects of the drug's diluent. Shook et al. (1984) described two cases of ethanol intoxication in patients receiving high doses of i.v. GTN. There has even been a report of a patient developing Wernicke's encephalopathy due to the ethanol and propylene glycol diluents in the GTN solution (Shorey et al., 1984). Presently, GTN formulations in nonalcoholic solutions are available.

X. Conclusion

The first description of GTN as a therapeutic agent for the treatment of angina pectoris appeared in 1879; since then it has remained an important drug in the treatment of angina together with other nitro compounds. In the present review we have discussed the

chemical nitrate esters, including their basic mechanism of action.

The therapeutic effect of nitrate esters is mainly due to their relaxant effect on vascular smooth muscles. Dilation of venous capacitance and arterial resistance vessels, as well as dilation of coronary arteries, is of importance. The organic nitrate esters have been observed to exert a number of biochemical actions that have been proposed to explain the pharmacological activity of these drugs. The emerging consensus strongly favours the intracellular second-messenger cGMP as the mediator of vascular smooth muscle relaxation elicited by organic nitrate esters.

Because of the physical and chemical properties, and hence the pharmacokinetic behavior, of nitro compounds, a number of factors have to be considered when conducting and interpreting pharmacokinetic studies of these substances. Pharmacokinetic studies of nitrate esters should include an estimation of active metabolites. Studies must be conducted under standardised conditions regarding posture, exercise, food intake, sampling, etc. The handling of samples is also important, as is the nature of the material used in the infusion sets. Studies should be performed on the actual patient group.

Considering the clinical therapeutic use of nitrate esters, sublingually administered GTN is still the cornerstone in the treatment of an attack of angina pectoris. A growing number of different formulations of GTN have been developed for prophylactic use. For prophylactic use, orally administered IS-5-MN or ISDN seems to be the best choice; dose titration studies have been more properly performed for these substances than for oral GTN. In certain patients, transdermal GTN might be a suitable, although expensive, alternative.

In the acute treatment of unstable angina and myocardial infarction, GTN given i.v. is of value in reducing symptoms. Further studies are required to ascertain the effects of nitrate esters in reducing complications and mortality.

There seem to be huge differences among patients regarding the risk of developing tolerance. Therefore, it is of value that every patient in a study be described individually and also that the results of the exercise test and other investigations be presented for each individual. In addition, it is important to have reasonable control of patient compliance because, for example, the ingestion of every tablet and ingestion at the correct time is essential when drawing conclusions regarding tolerance development.

Several experimental studies have shown that vascular smooth muscle tolerance to organic nitrate esters, induced either in vitro or in vivo, is accompanied by a reduced cGMP response. Several possible ways in which guanylate cyclase could be affected by tolerance induction were discussed.

As is apparent from this review, nitrate esters are, in general, well tolerated and have few side effects. The adverse effects that do exist are mainly attributed to the vasodilatory effects of the drugs.

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