The Transformation of Glyceryl Trinitrate and Other Nitrates by Glutathione-Organic Nitrate Reductase

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SUMMARY

The major route for glyceryl trinitrate transformation appears to be denitration in the presence of reduced glutathione (GSH). One molecule of glyceryl trinitrate reacts with two GSH to release one inorganic nitrite ion from either the 2- or 3-position to form 1,3- or 1,2-glyceryl dinitrate. With liver enzyme the glyceryl dinitrates and inorganic nitrite produced account for about 90% of the glyceryl trinitrate transformed. There is no product inhibition of the glyceryl trinitrate degradation reaction. The denitration of glyceryl dinitrate proceeds at only 2–5% of the rate for glyceryl trinitrate. Glyceryl mononitrate is practically unaltered by the liver enzyme. Thus, the denitration of glyceryl trinitrate does not lead to glycerol formation.

A rapid, sensitive enzymic assay based on the reaction of organic nitrates with GSH to yield GSSG was developed. The spectrophotometric or fluorometric method measures TPNH disappearance as the GSSG is catalytically reduced by glutathione reductase. Mannitol hexanitrate, erythrityl tetranitrate, and glyceryl trinitrate are rapidly transformed by the liver soluble enzyme in the presence of GSH, whereas isosorbide dinitrate and pentaerythritol tetranitrate are degraded at a slow rate by this system. The denitration of these compounds leads to a number of products with a lower number of nitrate ester groups. However, complete degradation of the parent molecule does not occur. The *in vivo* mechanism of organic nitrate detoxication appears to be similar to the enzymic denitration observed with liver glutathione-organic nitrate reductase.

INTRODUCTION

Oberst and Snyder (1) reported that glyceryl trinitrate (GTN) was metabolized to inorganic nitrite by rabbit liver homogenates. Heppel and Hilmoe (2) observed that GTN and erythrityl tetranitrate (ETN) reacted with reduced glutathione (GSH) to form oxidized glutathione (GSSG) and inorganic nitrite; this reaction was catalyzed by a hog liver enzyme. Hunter and Ford (3) demonstrated that less than one nitrite ion was formed per molecule of GTN metabolized. Needleman and Krantz (4, 5) found that the GTN transformation was accomplished by the formation of 1,2-glyceryl dinitrate (1,2-GDN) and 1,3-glyceryl dinitrate (1,3-GDN), both of which were observed to be relatively resistant to enzymic transformation and were identified as the urinary metabolites following the administration of GTN to rats. The glyceryl dinitrates were observed to be less active coronary vasodilators and blood pressure depressants than GTN. DiCarlo *et al.* (6, 7) studied the degradation of pentaerythritol tetranitrate (PETN) and observed partial denitration into its trinitrate, dinitrate, and mononitrate.

Denitration with the formation of in-

organic nitrite appears to be the only reported route of transformation for organic nitrates. This investigation is concerned with delineating qualitative and quantitative aspects of the reaction mechanism by which organic nitrates are denitrated by a soluble liver enzyme and by the whole animal.

MATERIALS AND METHODS

Measurement of enzyme activity. A rapid, sensitive enzymic assay of organic nitrate transformation was developed by Needleman and Hunter (8). The method is based on the scheme shown in Fig. 1. GTN

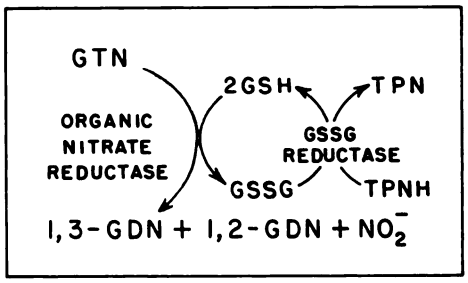

FIG. 1. *Schematic diagram of the GTN transformation reaction coupled with the GSSG reductase assay system*

Abbreviations employed are as follows: GTN, glyceryl trinitrate; GDN, glyceryl dinitrate; and NO_2, inorganic nitrite.

and GSH do react spontaneously at a slow rate to form inorganic nitrite and GSSG. The soluble liver enzyme, organic nitrate reductase, which greatly increases this reaction, was partially purified according to the method of Heppel and Hilmoe (2). Rall and Lehninger (9) demonstrated the specificity of glutathione reductase (GR) for GSSG in the presence of TPNH. In the assay GR and TPNH are added to the GTN–GSH system and GSSG formation is measured as TPNH disappearance in a Beckman DU spectrophotometer at 340 mμ. The spectrophotometric determination is performed at room temperature with 3 mM GTN, 3 mM GSH, 0.7 mM TPNH, GR, and liver enzyme. The reaction is carried out in 0.1 M phosphate buffer at pH 7.4. In order to minimize TPNH and GSH degradation by enzyme- or metal-catalyzed side reactions, 20 mM nicotinamide, 0.3 mM cyanide, 1 mM EDTA, and 0.02% bovine serum albumin were included in the medium.

The GR assay for organic nitrate reductase was adapted to fluorometry utilizing 10 μM TPNH and 1 mM GSH. Fluorometric measurements of the decrease in TPNH were made in a Farrand model A fluorometer with a Corning No. 5840 primary filter and a combination of Corning No. 4303 and 3387 for the secondary filter (10). Preincubation of GSH, TPNH, and GR removed contaminating GSSG from the GSH reagent. This preincubated solution was added to medium containing the organic nitrate, and the reaction was initiated with liver enzyme. The fluorometric assay was employed for comparison of the relative velocities with various organic nitrates as substrate for liver nitrate reductase. In these studies the organic nitrate concentration ranged from 0.004 mM to 2 mM in the presence of 1–4 μg protein of the partially purified enzyme. Maximum velocities, derived from Lineweaver-Burk plots, are reported as mmole·kg protein^{-1}·min^{-1}.

Analytical procedures. Inorganic nitrite was determined according to the method of Bell *et al.* (11) on mercuric chloride supernatants (12). Protein concentration was measured by the method of Lowry *et al.* (13). Total nitrate was measured with phenoldisulfonic acid according to DiCarlo *et al.* (6).

Animal experimentation. Male albino rats (Holtzman Rat Co., Madison, Wisconsin) weighing 150–175 g were fasted overnight but were allowed water ad libitum. The organic nitrates (10 mg) were administered intraperitoneally in 0.5 ml ethanol, and 5 ml of distilled water was administered by an oral feeding needle. The pooled urine of each treated group of three animals was collected at 24 hr, extracted with ether, concentrated, and chromatographed.

Chromatographic method. Analytical

thin-layer chromatography of organic nitrates was carried out on 0.25-mm silica gel using a solvent system of benzene: ethyl acetate:acetic acid 80:20:5 as previously described by Needleman and Krantz (5).

Preparative thin-layer chromatography on a 1.0-mm layer of silica gel was employed to separate the glyceryl dinitrates that were produced during 24-hr enzymic incubation of GTN in the presence of the GSH regenerating system.

Materials. Biochemical reagents and enzymes were obtained from Sigma Chemical Company; organic nitrates were the gifts of Atlas Chemical Industries, Inc., and Propellex Chemical Company; phenoldisulfonic acid was obtained from Harleco. Thin-layer chromatography supplies were obtained from Brinkman Instrument Company. All common chemicals were of analytical reagent grade.

RESULTS

Studies of the transformation reaction of glyceryl trinitrate (GTN) have been limited primarily to measurements of inorganic nitrite formation. With the observation that GTN is denitrated to 1,3-glyceryl dinitrate (1,3-GDN) and 1,2-glyceryl dinitrate (1,2-GDN), a more complete investigation of the GTN transformation can be undertaken.

The time course of the enzymic and the nonenzymic degradation of GTN and the appearance of 1,3- and 1,2-GDN is shown in Fig. 2. The curve for the enzymic disappearance of GTN is associated with a curve of similar rate for the formation of 1,3- and 1,2-GDN. The nonenzymic reaction of GTN and GSH produces a slow linear decrease of GTN and a linear production of 1,3- and 1,2-GDN. In the nonenzymic control incubation, equal quantities of both metabolites are produced, while in the enzymic reaction, the contribution of 1,3-GDN appears to be higher than 1,2-GDN. The difference in the level of the enzymic metabolites may reflect either preferential formation or more rapid transformation of one of the metabolites. During this 2-hr incubation only traces of mononitrate were found.

After 1 hr of incubation, 40% of the

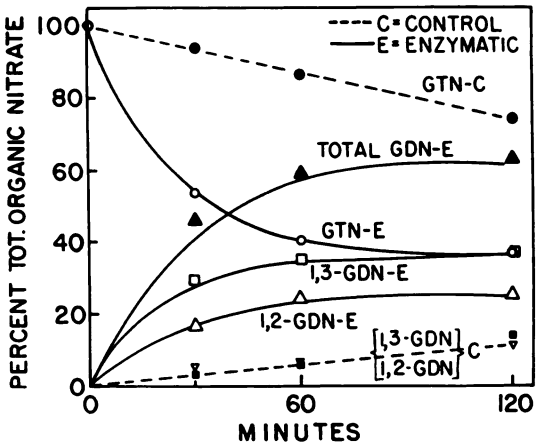

FIG. 2. *Time course of the disappearance of GTN and the appearance of 1,3- and 1,2-GDN*

The incubation was carried out at 37° in 0.1 M phosphate buffer, pH 7.4, containing GTN 4 mM, GSH 8 mM, TPNH 0.7 mM, GR, liver soluble enzyme—1.7 mg protein/ml, 20 mM nicotinamide, 0.3 mM cyanide, 1 mM EDTA, and 0.02% bovine serum albumin. Aliquots (2 ml) were taken at 0, 30, 60, and 120 min, extracted with ether, concentrated, and chromatographed. The spots were scraped from the plates and assayed with phenoldisulfonic acid for total nitrate. Values were normalized to correct for GDN, which is a contaminant of commercial GTN. Control incubations (C) contained no tissue.

original GTN remains and 60% of the total organic nitrate is present as 1,2- and 1,3-GDN. Since the reaction was started with 4 mM GTN, this indicates that 2.4 mmoles per liter of reaction mixture was transformed. During the 1-hr incubation, 1.7 mmoles of inorganic nitrite was formed per liter of reaction mixture. Thus 0.71 nitrite ion is recovered per GTN transformed. The total of the nitrate groups found in the 1,3-GDN and 1,2-GDN and appearing as inorganic nitrite accounts for about 90% of the nitrate groups in the GTN which has undergone enzymic reaction. Therefore, the major route of GTN degradation by the liver enzyme appears to be denitration with formation of the glyceryl dinitrates and inorganic nitrite.

Figure 2 demonstrates that the enzymic rate of GTN degradation and metabolite formation approaches zero after 1 hr. This could be the result of product inhibition, exhaustion of the reactants, or inactivation of the enzyme. The possibility of product inhibition was investigated and is shown in Fig. 3. No inhibition of the GTN transformation was exhibited following the addition of a mixture of 1,3- and 1,2-GDN or inorganic nitrite to the enzymic incubation. The increased rate of TPNH disappearance with GDN addition represents the sum of the nonenzymic and any enzymic transformation of these metabolites. The time course of GTN transformation was not altered by the addition of supplementary GSH. Preincubation of the enzyme with either substrate for 30 min did not substantially alter the reaction when the second substrate was added.

In the course of establishing the assay system for coupling organic nitrate transformation with GSSG reductase (Fig. 1), a comparison of the rate and stoichiometry of TPNH disappearance and nitrite formation was made (Fig. 4). Three incubation systems were employed so that the comparisons could be made in systems with the nonenzymic reaction, with liver soluble fraction, and with partially purified enzyme. In all three cases the TPNH consumption equaled the nitrite formation. This stoichiometry supports the coupling of liver glutathione–organic nitrate reductase and TPNH–glutathione reductase for assay purposes as shown schematically in Fig. 1. In the presence of GSSG reductase

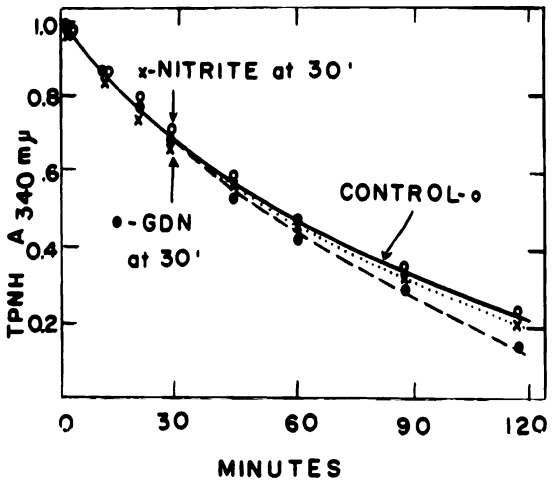

FIG. 3. *The effect of addition of products on the enzymic transformation of GTN*

The incubation mixture contained GTN 0.25 mM, GSH 4 mM, liver soluble enzyme—0.5 mg protein, other components as described in Fig. 2 and in the Materials and Methods section. TPNH utilization was followed at room temperature with direct readings at 340 mμ. Products were added at 30 min as follows: controls, nothing; nitrite, 100 μM; GDN, mixture of 1,2-GDN and 1,3-GDN 0.13 mM.

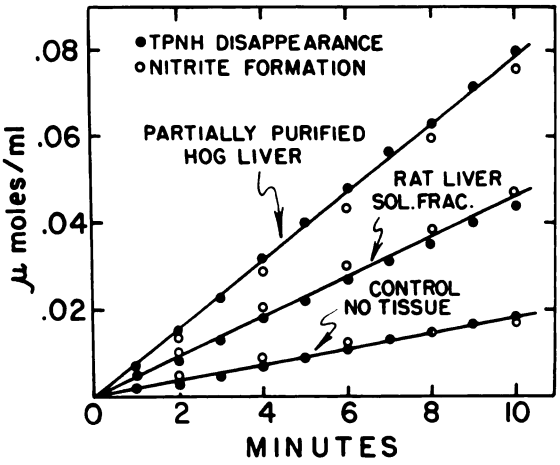

FIG. 4. *Comparison of the rates of TPNH disappearance and nitrite formation during GTN transformation*

Control, no tissue; partially purified hog liver, 50 μg protein; rat liver soluble fraction, 1.0 mg protein. The conditions for incubation are described in the Materials and Methods section.

and TPNH the GSH is regenerated and maintained at a constant level. There was no disappearance of TPNH or interference with fluorescence measurements with organic nitrates alone or in the presence of TPNH, GSSG, or GSSG reductase. All the alkyl nitrates tested show some nonenzymic reaction with GSH to form inorganic nitrite. This varies greatly with different compounds and ordinarily is deducted in calculating enzymic reaction rates.

Figure 5 represents the structure and a comparison of the maximum velocities of the enzymic transformation of various glyceryl nitrates, measured fluorometrically. The trinitrate ester is metabolized more than 20 times faster than the dinitrates, which are in turn metabolized more rapidly than the mononitrate by liver organic nitrate reductase.

A number of alkyl nitrates of varying onset and duration of action are employed in the treatment of angina pectoris. An investigation of the general transformation route of other alkyl nitrates in light of the present information regarding GTN was undertaken.

Figures 6 and 7 present the structures and the maximum velocities of transformation of a number of aliphatic nitrates by liver enzyme. Figure 6 includes a number of the nitrates employed therapeutically in the treatment of coronary insufficiency. Linear-chain polynitrate esters, such as mannitol hexanitrate (MHN), glyceryl trinitrate, and erythrityl tetranitrate (ETN)

GLYCERYL TRINITRATE	1,2-GLYCERYL DINITRATE
CH_2-ONO_2 $CH-ONO_2$ CH_2-ONO_2	CH_2-ONO_2 $CH-ONO_2$ CH_2-OH
120	5.0
1,3-GLYCERYL DINITRATE	**GLYCERYL MONONITRATE**
CH_2-ONO_2 $CH-OH$ CH_2-ONO_2	CH_2-ONO_2 $CH-OH$ CH_2-OH
2.5	0.7

FIG. 5. *Structures and rates of enzymic transformation of glyceryl trinitrate and its denitration products*

The enzymic activity at room temperature was measured fluorometrically as described under Materials and Methods. The number below each formula represents the V_{max} for the compound. The values are expressed as mmole·kg protein^{-1}·min^{-1} and are corrected for nonenzymic controls.

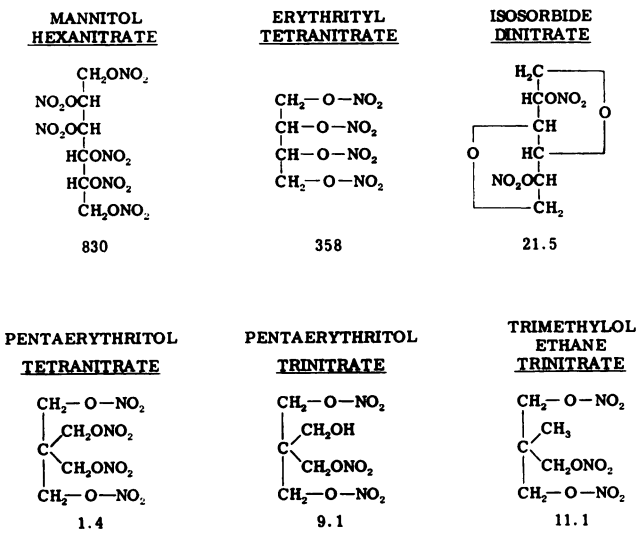

FIG. 6. *Structures and rates of enzymic transformation of some therapeutically employed alkyl nitrates and related compounds*

The enzymic activity at room temperature was measured fluorometrically as described under Materials and Methods. The number below each formula represents the V_{max} for the compound. The values are expressed as mmole·kg protein^{-1}·min^{-1} and are corrected for nonenzymic controls.

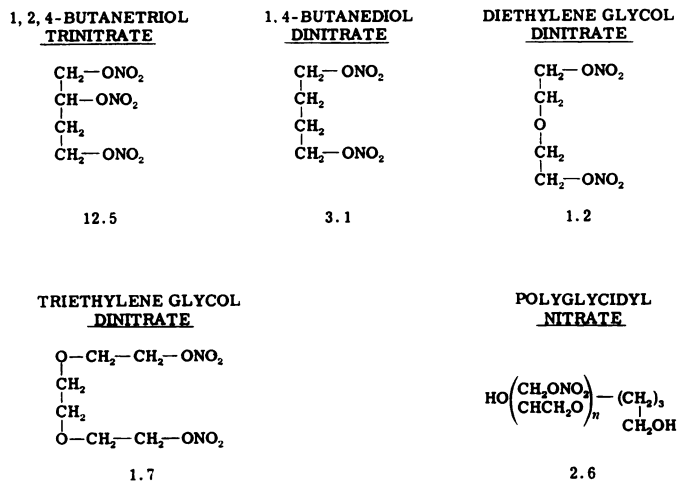

FIG. 7. *Structures and rates of enzymic transformation of some alkyl nitrates*

The enzymic activity at room temperature was measured fluorometrically as described under Materials and Methods. The number below each formula represents the V_{max} for the compound. The values are expressed as mmole·kg protein^{-1}·min^{-1} and are corrected for nonenzymic controls.

are rapidly transformed by the liver soluble enzyme in the presence of GSH. On the other hand, branched-chain alcohol nitrates such as pentaerythritol tetranitrate (PETN), pentaerythritol trinitrate (PETRIN), and trimethylol ethane trinitrate (TMETN) are slowly transformed by liver nitrate reductase (Fig. 6). Replacement of the nitrate group by hydrogen in a linear ester decreases the rate of

the enzymic reaction. This can be seen when ETN is compared to 1,2,4-butanetriol trinitrate (BTTN), or BTTN to 1,4-butanediol dinitrate (BDDN). Introduction of an ether linkage into a linear-chain nitrate compound further decreases the rate of enzymic transformation, as in the case of di- and triethylene glycol dinitrate.

Chromatographic analysis of the degradation of the principal organic nitrate vasodilators used therapeutically is shown after 4 hr of incubation with enzyme (Fig. 8). Earlier experiments demonstrated that these compounds oxidize GSH and release inorganic nitrite both enzymically and nonenzymically. In all cases chromatograms were run on nonenzymic control mixtures. In almost all cases the nonenzymic products were qualitatively similar to the products with enzyme. The following discussion is based on the quantitatively much larger yield of products seen with enzyme present.

With a chromatographic solvent system consisting of benzene:ethyl acetate:acetic acid 80:20:5 the greater the esterification the higher the migration on the plate. Thus the completely nitrated parent compounds move farther than the denitration products, a pattern which is reasonable in light of the increase in water solubility on replacing the nitrate ester with a hydroxyl. These chromatograms show only organic nitrates because the material applied represented the ether extracts of aqueous incubations. The color was developed with diphenylamine (17).

In Fig. 8 the spots nearest the top of the figure represent the parent compounds, as labeled. The GTN spot is followed by 1,2-GDN and 1,3-GDN, respectively. At 4 hr there is no detectable GMN, but a trace does appear after 24 hr. With mannitol hexanitrate (MHN) four definite metabolites and a tract (dashed circle) of another are present after 4 hr. After 24 hr seven products are present. However, the exact contribution of the enzymic and the nonenzymic reaction between 4 hr and 24 hr has not been determined. This situation differs from that with the GTN metabolites, which do not undergo further alteration. The transformation velocity of MHN,

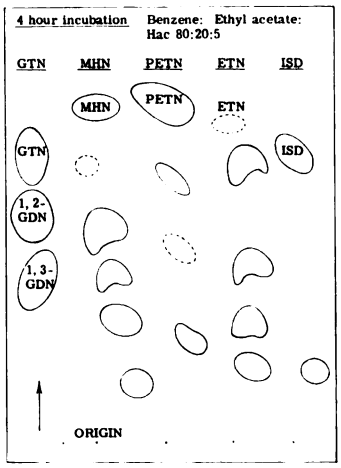

FIG. 8. *Chromatographic analysis of the 4-hr enzymic incubation of alkyl nitrates*

Incubations were carried out at 37° employing the following concentrations: glyceryl trinitrate (GTN) 4 mM, mannitol hexanitrate (MHN) 0.5 mM, erythrityl tetranitrate (ETN) 3.1 mM, pentaerythritol tetranitrate (PETN) 6.0 mM, isosorbide dinitrate (ISD) 8.8 mM, GSH 8 mM, liver soluble enzyme 1.7 mg protein/ml. Other ingredients as described under Materials and Methods. Aliquots were extracted with ether, concentrated, and chromatographed.

as measured by the fluorometric method, is about seven times faster than GTN (Fig. 5 and 6). The rate of MHN transformation now appears to represent the sum of the degradations of the parent compound and the metabolites as well. Pentaerythritol tetranitrate (PETN) shows large amounts of unchanged parent molecule and small amounts of three metabolites at 4 hr (Fig. 8), an observation that is in agreement with the low rate of enzymic transformation. According to DiCarlo (7), these metabolites are the tri-, di-, and mononitrates. The metabolite levels are diminishing at 24 hr, an observation which is consistent with the greater V_{max} for PETRIN (Fig. 6). Erythrityl tetranitrate (ETN) is metabolized three times faster than GTN (Figs. 5 and 6) with the appearance of large amounts of four organic nitrate metabolites at 4 hr (Fig. 8). The ETN metabolites do not appear to undergo much further degradation.

Isosorbide dinitrate is very slowly metabolized (Fig. 6), and this is reflected by large amounts of the parent molecule remaining at 4 hr. Two nitrated products appear.

The *in vivo* transformation of the vasodilator organic nitrates was determined by ether extraction of the pooled urine of rats treated with these compounds. The chromatogram of the urine extracts (Fig. 9)

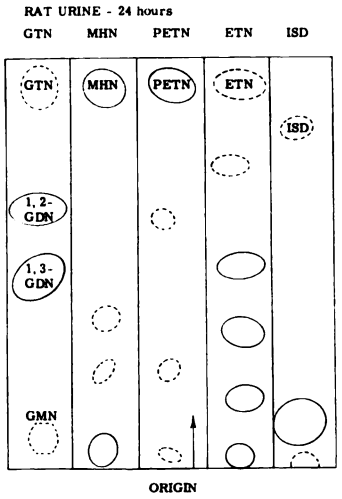

FIG. 9. *Chromatographic analysis of the pooled urine of rats treated with alkyl nitrates*

Experiment is described in the Materials and Methods section. Solvent system employed was benzene:ethyl acetate 4:1.

shows that some of the parent molecule of each compound appears in the urine along with an assortment of metabolites. The major metabolites of GTN are 1,3-GDN and 1,2-GDN, with a trace of GMN. The urine from MHN-treated rats shows one definite metabolite and traces of two others, an indication that the denitration of the metabolites continues *in vivo*. PETN is excreted with three metabolites. ETN is excreted with four major metabolites and a trace of another. This result agrees with the apparent stability of the ETN metabolites to further enzymic degradation. ISD is excreted primarily as one metabolite, with traces of the parent compound and of a second metabolite.

DISCUSSION

The enzymic degradation of GTN to the glyceryl dinitrates and inorganic nitrite accounts for about 90% of the nitrate groups in the GTN which has undergone reaction (Fig. 2). The finding that only 0.71 nitrite ion is recovered per GTN transformed in the longer incubations is in agreement with the earlier finding of Hunter and Ford (3) that less than one nitrate was found per molecule GTN degraded. Figure 4 demonstrates that in short experiments TPNH consumption equals the nitrate formation. This stoichiometry supports the coupling of the enzymes as presented schematically in Fig. 1. It is apparent that for each nitrate formed, two GSH are oxidized. This means that one molecule of GTN reacts with two GSH to release one inorganic nitrite ion from either the 2- or 3-position to form 1,3- or 1,2-GDN. The 1:1 yield of inorganic nitrite from GTN in these short incubations (up to 10 min) indicates that the lower nitrite values found in the longer-term experiments are due to side reactions, such as oxidation of nitrite to nitrate (14).

Since TPNH can be measured directly and continuously either spectrophotometrically or fluorometrically, the assay system shown in Fig. 1 appears to be superior to the various colorimetric assays for inorganic nitrite, which require removal of aliquots, precipitation of protein, centrifugation, and addition of reagents to develop the color.

The slow rate of GDN degradation indicated by the low V_{max} (Fig. 5) is consistent with the finding of Needleman and Krantz (5) that the dinitrates of glycerol appeared as the primary urinary metabolites following GTN administration to rats. The fact that GMN is practically unaltered is in agreement with the finding of Heppel and Hilmoe (2) that no glycerol was found after GTN degradation. The metabolism of 1,2-GDN proceeds somewhat more rapidly than 1,3-GDN. This difference may account for the unequal levels of these metabolites accumulating in the enzymic experiment shown in Fig.

2. A mixture of 1,2- and 1,3-GDN did not compete effectively with GTN in the enzymic reaction, otherwise there would have been inhibition of GTN transformation in the experiment shown in Fig. 3.

The relation of the enzymic transformation of GTN to the short duration of action of this compound is an important question. The GDN metabolites were previously shown by Needleman and Krantz (5) to be less active than the parent molecule. The resistance of the metabolites to degradation, apparent in the enzyme studies (Figs. 2 and 5) and in the fact that they appear in the urine, does not manifest itself in a longer therapeutic response. The transient duration of action of GTN *in vivo* may depend in part on liver organic nitrate reductase, but short action with preparations such as isolated smooth muscle indicates that other mechanisms must be involved.

Linear-chain polynitrate esters were shown to be rapidly transformed by liver glutathione–organic nitrate reductase, whereas branched-chain alcohol nitrates were only slowly degraded (Fig. 6). O'Meara *et al.* (15) found that the degree of nitration influenced vasodilator activity of glucose derivatives. They showed that mono- or dinitrate glucosides were devoid of activity while tri- and tetranitrate glucosides possessed activity. All active vasodilators that they studied had at least one pair of adjacent carbons nitrated. Figure 5 illustrates that replacement of a nitrate ester group with a free hydroxyl decreases the velocity of enzymic degradation, since GTN is transformed much more rapidly than GDN, and GDN more rapidly than GMN. In addition, if the nitrate group is replaced by hydrogen in a linear ester the rate of the enzymic reaction decreases. The branched-chain compound PETN represents an exception to these generalizations. This might be the result of the very low solubility of PETN in the experimental system. Anhydride formation, as in isosorbide dinitrate, markedly decreases enzymic hydrolysis. Krantz *et al.* (16) tested a series of nitrates of sugar alcohols and their anhydrides and found that the ether linkage increased water solubility, decreased blood pressure-lowering activity, increased duration of action, and decreased *in vivo* hydrolysis of the anhydrides.

The denitration of GTN leads to the more water-soluble dinitrates and traces of mononitrate (5). The appearance of three nitrate metabolites in the urine of PETN-treated rats indicates *in vivo* degradation of this compound (Fig. 9). DiCarlo (7) has presented evidence for enzymic transformation of PETN in the erythrocytes to the tri-, di- and mononitrate. It will be recalled that the erythrocytes are rich in GSH (18), so that final determination of the contribution from enzymic and non-enzymic reactions requires further study. Isosorbide dinitrate is slowly transformed to two nitrate products (Fig. 8). These are very probably mononitrates, but since ISD is a symmetrical molecule the formation of two metabolites indicates that an additional change such as ring cleavage must be responsible.

The results presented in this paper indicate that the *in vivo* mechanism of detoxification of organic nitrates is similar to the enzymic denitration observed with liver organic nitrate reductase. This reaction leads to formation of inorganic nitrite, which does relax smooth muscle *in vitro*. However, Krantz *et al.* (19–21) and Needleman and Krantz (5) have demonstrated that the vasodilator activity of organic nitrates probably resides in the parent molecule and does not seem to be related to their transformation to nitrite in the body. The enzymic denitration in the body results in formation of products with greater water solubility.

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