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ROLE OF ENDOGENOUS GLUTATHIONE IN THE METABOLISM OF GLYCERYL TRINITRATE BY ISOLATED PERFUSED RAT LIVER*

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Abstract—Glyceryl trinitrate (GTN) metabolism in perfused rat livers: (1) proceeded extremely rapidly with a half-time of approximately 1 min (in recirculating perfusions); (2) was drastically inhibited (98 per cent) by bromobenzene pretreatment *in vivo* of rats; (3) was enhanced (42 per cent) by phenobarbital *in vivo*; and (4) was not altered by GTN tolerance. In non-recirculating experiments, GTN metabolism did not proceed at a constant rate, and the livers were only capable of degrading a fixed amount of GTN (about 70 m-moles/kg wet weight of liver). High concentrations of GTN perfused through the isolated rat liver resulted in an 85 per cent depletion of endogenous liver glutathione and an 80 per cent decrease in ATP. These results are hypothesized to be consistent with the fact that GTN metabolism in perfused rat livers appears to be dependent on the endogenous glutathione (GSH) reserve. High concentrations of GTN decrease liver ATP by inhibiting mitochondrial phosphorylation. The low ATP interferes with GSH synthesis and results in a net depletion of liver GSH and therefore a decreased ability to continuously degrade GTN.

A GLUTATHIONE-dependent enzyme isolated from the soluble fraction of liver homogenates has previously been reported to metabolize glyceryl trinitrate (GTN).[1,2] GTN has also been shown to undergo a nonenzymatic reaction with glutathione (GSH).[3] In this investigation the relationship of liver glutathione to GTN metabolism was studied in isolated perfused rat livers. Such perfusion experiments permit evaluation of total liver GTN degradation (not merely the GSH-dependent reaction) as well as simultaneous consideration of the effect of GTN on some endogenous liver metabolites. In addition, by perfusing the livers without blood, the complication of nonenzymatic degradation due to the presence of GSH in erythrocytes is removed.

METHODS AND MATERIALS

Liver perfusion. Unfasted rats (Holtzman, approx. 250 g) were anesthetized with 35 mg/kg pentobarbital. The peritoneal cavity was opened and the animals were heparinized by direct injection (100 USP units) into the inferior vena cava. The bile duct and portal vein were cannulated (polyethylene tubing, sizes PE 10 and 205 respectively). The thorax was opened, the inferior vena cava was cut, and the liver was immediately (within 15 sec after occlusion of the portal vein) perfused by way of the portal vein cannula, thereby minimizing the initial period of ischemia. The thoracic vena cava was cannulated (PE 205) and the liver was cut free of the animal and placed

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in the temperature-controlled perfusion apparatus (Metro-Scientific). The livers were perfused for 10 min with Krebs-bicarbonate solution [bubbled with O_2–CO_2 (95–5% which maintained a pH of 7·4) at 37°] and then switched to drug-containing solution. Two types of perfusions were carried out. In one set of experiments, 10 ml of radioactive drug-containing medium was continuously recirculated through the isolated liver for 10 min. In the non-recirculating experiments, fresh medium was perfused through the liver and the effluent fluid was discarded. The composition of the perfusion medium included: 120 mM NaCl, 4·7 mM KCl, 2·5 mM anhydrous $CaCl_2$, 1 mM $MgSO_4 \cdot 7H_2O$, 1 mM KH_2PO_4, 20·5 mM $NaHCO_3$ and 10 mM glucose. The perfusion pressure was 30 cm water and the flow rate was 40 ml/min throughout the experiment.

Various pretreatments *in vivo* were performed before liver perfusion in order to alter GTN metabolism. The isolated rat livers were subsequently perfused with medium (which was not recirculated) containing 1 mM GTN. The pretreatment conditions were as follows: (1) phenobarbital sodium (1 mg/ml) was added to the rats' drinking water for 2 weeks before the experiment; (2) bromobenzene (1·5 g/kg in cottonseed oil) was administered (i.m.) once daily for 2 successive days; and (3) rats were made tolerant to GTN by the administration (s.c.) of 100 mg/kg three times daily for 4 days.

GTN and NO_2^- analysis. Aliquots of perfusion solution were extracted twice with 5 ml petroleum ether which quantitatively removed GTN from its metabolites [glyceryldinitrate (GDN), glyceryl mononitrate (GMN) and NO_2^-]. The petroleum ether fractions were combined and allowed to evaporate. The GTN was hydrolyzed to inorganic nitrite by heating at 105° for 1 hr with 1 ml of 0·5 N KOH. The tubes were cooled to room temperature and the following reagents were added (with mixing after each addition): 1 ml of 4 N HCl, 1 ml of 0·2% procaine hydrochloride, and 1 ml of 0·1% *N*-(1 naphthyl)-ethylene-diamine dihydrochloride. Samples were read after 10 min at 548 mμ.

Lactate–pyruvate. Lactate–pyruvate concentrations in the perfusion media were measured to determine the viability of the isolated liver preparations. Analyses of lactate or pyruvate were made fluorometrically by measuring changes in the concentration of NADH in the presence of lactic dehydrogenase.[4] Lactate–pyruvate ratios of 10–11 have been reported by Burch *et al.*[5] *in vivo* and by Exton and Park[6] and Papenberg *et al.*[7] in rat livers perfused with blood. The ratios obtained in livers perfused (by medium that was continuously recirculated) with or without drug were less than 10 for up to 30 min, indicating that the tissues were adequately oxygenated (Table 1). In non-recirculating perfusion experiments the lactate/pyruvate ratios remained below 10 for 50 min and bile production (at a rate of 0·5–1·0 ml/hr) continued throughout the experiment, thereby indicating the viability of the preparations.

Endogenous metabolite extraction. Perfused livers were rapidly frozen in liquid nitrogen at the appropriate time. Two samples (about 100 mg) were dissected (in a −20° room) from the frozen liver using a single-edged razor blade. After weighing, the tissues were transferred to a −10° alcohol–dry ice bath and were ground (with glass rods) to a uniform paste in 3 M $HClO_4$. The extracts were neutralized with 2 M $KHCO_3$ and centrifuged. The supernatant fraction containing the metabolites was stored at −80°.

Total glutathione. Liver extracts (60 μl equivalent to 3 mg) were added to 1 ml of

reagent solution which contained: 0·1 M phosphate buffer, pH 7·4; 20 mM nicotinamide; 3 mM EDTA; and 0·2% bovine serum albumin. For total glutathione determinations (expressed in terms of GSH), the following sequence of additions was employed: 1 μl of 30% H_2O_2 incubated 15 min at room temperature (to convert GSH to GSSG); 30 μg catalase (until bubbling stopped); and 1 μl of 4 mM NADPH (final concentration, 4 μM). All fluorometric measurements were made in a Farrand fluorometer with a Corning No. 5840 primary filter and a combination of Corning No. 4303 and 3387 as the secondary filter. The samples were read, 0·2 μg of glutathione reductase was added, and a final reading was made when the reaction was complete The total GSH content was obtained by doubling the GSSG content, which is equivalent to the decrease of NADPH fluorescence in this assay.

TABLE 1. LACTATE/PYRUVATE IN MEDIA PERFUSING ISOLATED RAT LIVER PREPARATIONS*

Perfusion time (min)	Lactate (μmoles/l.)	Pyruvate (μmoles/l.)	Lactate/pyruvate ratio
−10	394 ± 54	85 ± 17	4·6
−5	453 ± 120	111 ± 40	4·1
GTN (1 mM)			
1	216 ± 49	43 ± 14	5·0
5	470 ± 88	68 ± 20	6·9
10	703 ± 81	104 ± 26	6·7
15	864 ± 178	137 ± 33	6·3
30	1752 ± 475	190 ± 34	9·2

* Krebs-bicarbonate solution (100 ml) was recirculated through a rat liver for 10 min of equilibration. After passing through the liver, the medium was pumped to a reservoir where it was continuously aerated (O_2–CO_2, 95–5%). The liver was then similarly perfused with 100 ml of a 1 mM GTN solution. Aliquots were removed at the indicated times. Values are mean ± S.E. ($n = 4$).

Adenosine triphosphate. Liver ATP determinations were performed on the neutralized $HClO_4$ extracts by measuring the appearance of NADPH fluorescence after the addition of hexokinase and glucose-6-phosphate dehydrogenase.[4]

Materials. GTN-1,3-^{14}C was synthesized from glycerol-1,3-^{14}C (New England Nuclear) by the nitration procedure of Lawrie[8] and purification was accomplished according to the method described by Dunstan *et al.*[9]

The enzymes employed in the assays were obtained commercially and included beef heart lactic dehydrogenase (Worthington Biochemical Company), yeast glutathione reductase (Sigma Chemical Company), beef liver catalase, yeast hexokinase, and yeast glucose-6-phosphate dehydrogenase (Boehringer and Sons). The standard substrates (lactate, pyruvate, GSH, GSSG, ATP, etc.) were obtained from the Sigma Chemical Company. The unlabeled GTN was extracted from a 10% (w/w) mixture (i.e. each 100 g powder contained 10 g GTN; Atlas Chemical Company).

RESULTS

Recirculation experiments

GTN-1,3-^{14}C was continuously recirculated through a perfused rat liver for 10 min (Fig. 1). Aliquots of perfusion solution were extracted, then separated on thin-layer plates, scraped and counted. Thin-layer chromatography (TLC) was carried out on silica gel plates with a solvent system of benzene–ethyl acetate (4:1). The R_f values were as follows: GTN, 0·81; 1,3-GDN, 0·51; 1,2-GDN, 0·42; GMN, 0·11. The disappearance of the parent compound was extremely rapid with a half-time of approximately 1 min and 90 per cent loss by 5 min. The glyceryl dinitrates rose rapidly to a plateau between 2 and 10 min. (The two GDN isomers were produced in equal amounts.) After an initial lag period, the glyceryl mononitrate concentration rose slowly but steadily throughout the perfusion period. The total of the GDN and GMN accounted for the GTN metabolized.

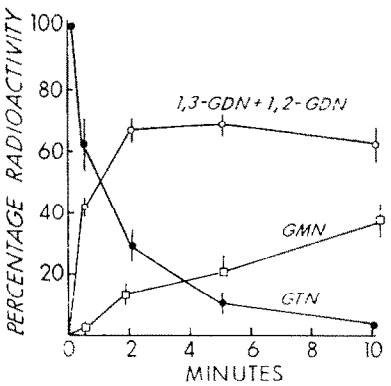

FIG. 1. Perfusion of rat liver with a recirculating solution of GTN. The livers were equilibrated with Krebs-bicarbonate (37°) for 10 min. The drug solution (10 ml) containing 0·2 μc GTN-1,3-^{14}C in 1 mM unlabeled GTN was then recirculated through the liver for 10 min. Aliquots of perfusion media (1 ml) were taken at the indicated times for chromatographic separation and for inorganic nitrite determinations. A 0·5-ml portion of the aliquot was extracted with ether, air dried and quantitatively separated on silica gel plates (solvent system: benzene–ethyl acetate, 4:1). The plates were sprayed with diphenylamine and scanned on a Vanguard Autoscanner 880. The spots were scraped and counted in a Packard Tri-Carb 3375 liquid scintillation counter. Values are expressed as the percentage of the total counts recovered at each time interval (± S.E.). The levels of 1,3-GDN and 1,2-GDN were equal throughout the experiment and therefore were added together.

Ether extracts of rat liver taken after GTN perfusion (10 min) were chromatographed on silica gel plates. The material contained very low GTN (4 ± 1%, mean ± S.E.) and mostly glyceryl dinitrates (56 ± 15%) and GMN (40 ± 13%, $n = 5$).

Inorganic nitrite formation followed the pattern of GTN disappearance. At 0·5, 2, 5 and 10 min, the NO_2^- concentration in the media from livers perfused with 1 mM GTN was 0·10 ± 0·02, 0·48 ± 0·06, 0·74 ± 0·06, and 0·88 ± 0·08 mM ($n = 6$) respectively. Thus by 10 min there was approximately 1 mole of NO_2^- liberated per mole of GTN metabolized. A 1:1 yield of NO_2^-–GTN in nonenzymatic reactions between GTN and GSH and in GSH-dependent reactions catalyzed by either partially purified hog liver homogenate or by rat liver 100,000 *g* supernatants was previously demonstrated.[2] Although NO_2^- (1 m-mole/l.) perfused through isolated rat liver

preparations was quantitatively recovered (data not shown), some loss of intracellular NO_2^- (produced by GTN degradation) by side reactions must occur in order to account for the GMN formation.

Perfusion without recirculation

Varying GTN concentrations. The rapid exponential disappearance of GTN in recirculating perfusions makes it difficult to study any drug interactions which might enhance the rate of metabolism. The recirculating experiments demonstrated that the total of the GDN and GMN produced was equivalent to the amount of GTN degraded and that there was no liver uptake of GTN. Therefore, in subsequent experiments GTN disappearance was used as the measure of GTN metabolism. In the remaining experiments the livers were perfused without recirculation with a fixed concentration of GTN (unlabeled).

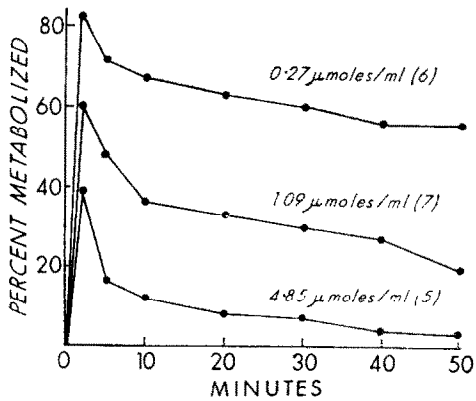

FIG. 2. Non-recirculating rat liver perfusions with different GTN concentrations. Aliquots of perfusion effluent were taken at the indicated times. GTN was quantitatively extracted and hydrolyzed to inorganic nitrite for assay (see Methods). Values are expressed as percentage of GTN which disappeared from the perfusion medium. The GTN concentrations of the solutions entering the livers are indicated on the curves and the number of animals is indicated in parentheses.

Three different concentrations of GTN (covering a 20-fold range) were used (Fig. 2). The liver preparations were unable to maintain a constant rate of GTN metabolism. In all cases there was a peak response at 2 min (earliest time measured) followed by a steady decline in the rate of metabolism. The lowest percentage metabolism was observed with the highest GTN concentration (4·85 mM, the limit of solubility); from 10 to 50 min the livers metabolized only 10 per cent or less of the drug. The highest percentage disappearance occurred with the lowest level (0·27 mM) and in this case there was only a moderate fall in rate with time. In contrast to these percentage differences, the total amount of GTN metabolized by the livers was nearly independent of the perfusion concentration. Livers perfused for 50 min with 4·85, 1·09 and 0·27 mM GTN degraded 78, 67 and 66 (projected for 100 min) m-moles/kg wet weight respectively.

Needleman and Hunter[2] reported a V_{max} for glutathione-organic nitrate reductase in rat liver of 1 mole/kg of liver/hr. The isolated rat liver perfused with a GTN concentration of 4·85 μmoles/ml metabolized about 40 per cent of the drug at the 2-min

sampling time (Fig. 2). Thus the 10 g liver degraded 78 μmoles/min/10 g of liver [4·85 μmoles/ml × 40 ml/min (flow rate) × 40%] or an initial rate of 0·47 moles GTN metabolized per kg of liver per hr. The rate of GTN metabolism was declining very rapidly during the early time period of the perfusion and the initial rate of degradation must have been considerably higher. It would therefore appear that the GTN metabolism of the intact perfused liver is primarily dependent on the glutathione-organic nitrate reductase activity.

Effect of pretreatment on GTN metabolism. Rats were treated in various ways *in vivo* before liver perfusion in an effort to alter the rate of GTN degradation. All livers were subsequently perfused with 1 mM GTN for 50 min (Fig. 3).

Chronic administration *in vivo* of phenobarbital accelerated the rate of GTN metabolism by liver perfusion. The amount of GTN metabolized in 50 min by the livers from phenobarbital-pretreated rats was 95 ± 9 m-moles/kg wet weight of liver (mean ± S.E., $n = 6$), which represents a 42 per cent increase over controls; the net increase was statistically significant ($P < 0.05$). The total amount of GTN metabolized was also greater because phenobarbital induced a 25 per cent increase in liver weight. Phenobarbital pretreatment of rats was previously demonstrated to cause a 20 per cent enhancement of GTN metabolism by liver homogenates.[1] Bogaert *et al.*[10] found that phenobarbital pretreatment of rabbits accelerated excretion of metabolites after GTN administration.

Tolerance to the cardiovascular effects of GTN can be induced by repeated administration of GTN.[11,12] In the present investigation there was no difference in the degradation of GTN by livers from tolerant or control rats (Fig. 3). Needleman[11] previously reported no enhancement of glutathione-organic nitrate reductase nor any

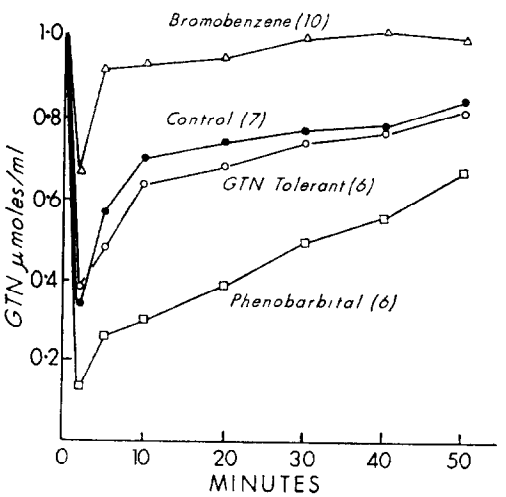

FIG. 3. Comparison of various pretreatment conditions *in vivo* on the disappearance of GTN from perfused rat livers. The pretreatment conditions were as follows: (1) phenobarbital sodium (1 mg/ml) was added to the rats' drinking water for 2 weeks; (2) bromobenzene (1·5 g/kg) dissolved in cottonseed oil was administered (i.m.) once daily for 2 successive days; and (3) rats were made tolerant to GTN by the administration (s.c.) of 100 mg/kg three times a day for 4 days. Values represent the concentration of GTN remaining in the effluent medium which continuously perfused the rat liver. The concentration of GTN in the perfusion solution was 1·09 mM. Parentheses indicate the number of animals.

changes in the concentration and pattern of urinary metabolites from tolerant rats after GTN administration.

Bromobenzene pretreatment caused a marked inhibition of GTN degradation with only 6 ± 0·2 m-moles/kg (mean ± S.E.) of GTN or a 90 per cent decrease compared to controls; the difference was highly significant ($P < 0·001$). There were no gross pathological changes noted in the rat livers removed from the bromobenzene-treated animals.

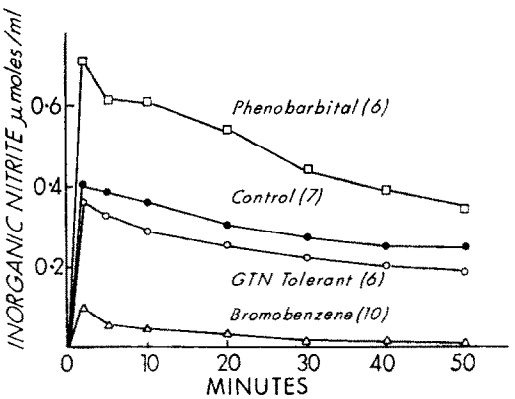

FIG. 4. Comparison of various pretreatment conditions *in vivo* on the appearance of inorganic nitrite from isolated rat livers perfused with GTN. Conditions of the experiments are in the legend for Fig. 3.

The effect of the various pretreatments was borne out by measuring inorganic nitrite as the end product of GTN degradation (Fig. 4). The highest rate of nitrite formation occurred with phenobarbital pretreatment, whereas there was virtually no denitration of GTN by livers from bromobenzene-treated animals. Finally, there was no significant difference between controls and livers removed from GTN-tolerant animals.

Endogenous glutathione and ATP levels after liver perfusion

$HClO_4$ extracts of perfused livers were assayed for total glutathione content and ATP (Table 2). The GSH concentration in "zero time" controls agrees with the level reported by Matschinsky *et al.*[13] in rat livers perfused with blood. Perfusion of the liver with medium alone led to a depletion of one-third of the total GSH. The loss of liver GSH was greatly accelerated when the livers were perfused with increasing concentrations of GTN. Fifty min of perfusion with 4·5 mM GTN led to an 85 per cent decrease in liver GSH. Pretreatment with phenobarbital produced no significant change in GSH levels. Bromobenzene pretreatment caused a 70 per cent decrease in GSH. This small reserve of GSH was nearly completely depleted (95 per cent) by perfusion with GTN.

The ATP concentration in "zero time" controls was found to be about one-third lower than levels found *in situ*.[5] Perfusion without drug for 50 min or pretreatment with phenobarbital did not alter the ATP levels. When isolated rat livers were perfused with GTN, a concentration-dependent depletion of ATP was observed (Table 2).

TABLE 2. LIVER GLUTATHIONE AND ATP CONCENTRATIONS MEASURED BEFORE AND AFTER PERFUSION WITH GTN*

Animal pretreatment	Perfusion conditions	Total GSH (m-moles/kg wet)	ATP (m-moles/kg wet)
None (4)	"Zero" time control, no drug	3·42 ± 0·43	1·90 ± 0·13
None (3)	50 min, no drug	2·44 ± 0·67	1·84 ± 0·05
None (3)	50 min with 0·25 mM GTN	1·27 ± 0·31	1·86 ± 0·29
None (4)	50 min with 1·0 mM GTN	0·97 ± 0·05	1·33 ± 0·05
None (3)	50 min with 4·5 mM GTN	0·46 ± 0·07	0·39 ± 0·09
Bromobenzene (2)	"Zero" time control, no drug	1·10, 1·08	1·44, 1·40
Bromobenzene (7)	50 min with 1·0 mM GTN	0·13 ± 0·02	0·69 ± 0·10
Phenobarbital (2)	"Zero" time control, no drug	3·40, 3·48	1·89, 2·13
Phenobarbital (2)	50 min with 1·0 GTN	1·01, 1·05	1·54, 1·45

* Experimental conditions are in the legends for Figs. 2 and 3. The "zero time" sample was taken 5 min after the liver was perfused with Krebs-bicarbonate medium to wash the liver free of blood. Parentheses indicate the number of livers analyzed. The values represent the means ± S.E.

Normal livers exposed to 50 min of 4·5 mM GTN exhibited an 80 per cent decrease in ATP. Smaller changes in ATP content were noted in normal, phenobarbital-pretreated, or bromobenzene-pretreated rat livers perfused with 1 mM GTN.

DISCUSSION

GTN metabolism in perfused rat livers appears to be dependent on the endogenous GSH reserve. This hypothesis is supported by the following observations: (1) GTN metabolism did not proceed at a constant rate and the livers were capable of degrading only a fixed amount of drug, thereby implying the depletion of some endogenous component; (2) bromobenzene administration, which has been demonstrated to deplete liver GSH,[14] drastically inhibited GTN metabolism; and finally (3) direct measurements of endogenous liver GSH levels indicated disappearance of GSH during GTN metabolism. The small reserve of GSH remaining in liver after bromobenzene pretreatment was completely depleted by drug perfusion and paralleled the observed extremely low rate of GTN degradation.

In the recirculating liver perfusion there was an extremely rapid GTN disappearance. In non-recirculating experiments (during the first 2 min) more than 80 per cent of the GTN (0·25 mM) was degraded in one pass through the liver. Constant infusion of GTN at lower concentrations or single bolus administration of larger doses would probably be degraded in one pass through the liver. It is therefore fortunate that GTN is taken sublingually by humans. This route of administration delivers the drug to the heart and coronary system directly without initially passing through the liver as would occur if the drug was taken orally. Bogaert *et al.*[15] administered GTN to rabbits whose livers were excluded from the blood circulation and noted a greatly prolonged peak in nitrate plasma levels.

GSH synthesis from its component amino acids is an energy-consuming process in which two molecules of ATP are required per molecule of GSH synthesized.[16,17] Bromobenzene pretreatment caused a 70 per cent fall in liver GSH but only a 25 per cent decrease in ATP (Table 2). The small decrease in ATP induced by bromobenzene

would not be expected to interfere with GSH synthesis. Consistent with this point is the observation by Kalser and Beck[14] that bromobenzene did not interfere with net synthesis of liver GSH in mice. The depletion of GSH was accounted for by Booth *et al.*[18] who demonstrated that bromobenzene was conjugated with liver glutathione and excreted after kidney hydrolysis as S-(*p*-bromobenzyl)-cysteine.

The ability of GTN perfusion to decrease liver ATP concentrations is consistent with the observation that organic nitrates alter the oxygen consumption of rat liver mitochondria.[19,20] GTN was shown to produce minimal effects at 67 μM, but produced complete loss of respiratory control at 530 μM.[19] Boime and Hunter (personal communication) demonstrated that GTN (at least 1 mM) inhibited phosphorylation at the level of NADH dehydrogenase in rat liver mitochondria. After 50 min of perfusion of the isolated rat liver with 1 mM GTN, a moderate decrease in liver ATP concentration was noted (Table 2) at a time when the concentration of GTN in the perfusate was reduced to 0·2–0·3 mM (calculated from Fig. 2). But a drastic decrease in liver ATP was produced by perfusion with 4·85 mM GTN.

The synthesis and breakdown of GSH in liver is a rapid process. The turnover rate of GSH in rat liver has been reported by a number of workers to be about 2 hr.[21] A unifying hypothesis developed to explain this collection of data is illustrated in Fig. 5. The isolated rat liver is perfused with GTN, which oxidizes GSH and degrades the GTN to its lower nitrates. The GSSG is reduced rapidly by glutathione reductase and NADPH. With increasing concentration of GTN in the perfusion medium, the isolated rat liver was found to be incapable of maintaining a constant rate of GTN degradation. The high concentration of GTN caused a decrease in ATP resulting from the inhibition of mitochondrial phosphorylations. The low ATP in turn would interfere with the synthesis of GSH, whereas the degradation process rapidly continues, resulting in a net depletion of liver GSH. The level of GSH becomes limiting and the perfused liver possesses a decreased ability to degrade GTN continuously. Bromobenzene, on the other hand, does not interfere with GSH synthesis but rather it enhances GSH depletion by conjugation.

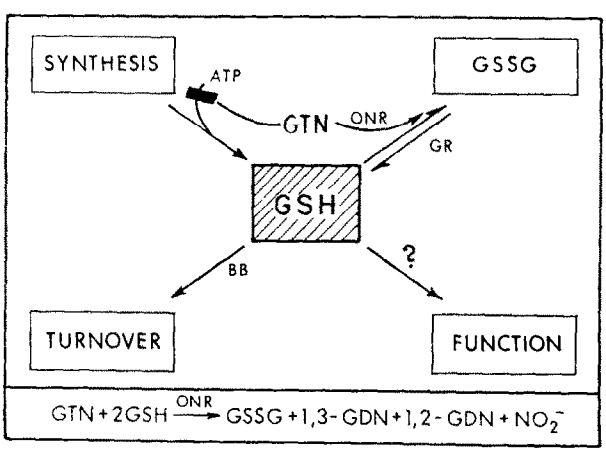

FIG. 5. Schematic diagram of the interrelationship between GTN transformation and endogenous liver GSH. Abbreviations: ONR, organic nitrate reductase; GR, glutathione reductase; BB, bromobenzene.

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