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THE BIOTRANSFORMATION OF NITROGLYCERIN*

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Abstract—The transformation of nitroglycerin (NG) was accomplished by the formation of 1,2- and 1,3-dinitroglycerin. The dinitrates were resistant to further degradation and appeared as the urinary metabolites after the administration of NG. The dinitroglycerins were less active coronary vasodilators and blood pressure depressants than NG. A heat-stable inhibitor of the spontaneous reaction between NG and glutathione (GSH) was removed by dialysis. The enzyme that catalyzes the NG–GSH reaction was not found in heart but was localized in rat liver soluble fraction and was enhanced by phenobarbital pretreatment.

OBERST and Snyder[1] reported that nitroglycerin (NG) was partially metabolized to inorganic nitrite by rabbit liver homogenates. Heppel and Hilmoe[2] observed that NG reacted with reduced glutathione (GSH) to form oxidized glutathione and inorganic nitrite; the rate of nitrite formation was enhanced by a hog liver enzyme in the presence of cyanide. Since inorganic nitrate and GSH did not react spontaneously, these authors deduced that nitrate ester reduction preceded hydrolysis. Hunter and Ford[3] accounted for less than one nitrite ion per molecule of NG metabolized. The position of the liberated nitrite was unknown, and no consideration of other metabolic products has been undertaken.

This investigation is concerned with some aspects of the transformation of NG, the nature and activity of the resulting metabolites, and the relationship between the transformation and the therapeutic mechanism of action of organic nitrates.

METHODS

Enzymatic transformation of nitroglycerin

A 7% (w/v) suspension of the hog liver or heart acetone powder in 0·067 M phosphate buffer (pH 7·4) was freshly prepared and centrifuged before each experiment. Inactivation was accomplished by boiling for 5 min and was followed by centrifugation.

Rat liver homigenates[4] were prepared with 0·25 M sucrose in either 0·01 M Tris buffer or 0·067 M phosphate buffer at pH 7·4. Differential centrifugation of mitochondria was performed according to the method of Schneider[4] in an International PR-1 centrifuge; the microsomal fraction was prepared in a Beckman Spinco ultracentrifuge model L at 100.000 *g* for 90 min.

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Phenobarbital induction was achieved by injecting 200-g male rats with phenobarbital sodium, 10 mg/10 ml per kg in saline, i.p., twice daily for four days prior to the test. Control animals followed the same schedule but received only saline solution, 10 ml/kg. The animals were killed with a guillotine decapitator, and the liver homogenates were prepared.

Incubations were performed in 1 ml 0·067 M phosphate buffer containing: 4 μmoles NG, 8 μmoles GSH, 8 μmoles cyanide, and 0·5 ml tissue preparation. Control tubes were incubated without tissue. Incubations were performed aerobically at 37° for 10 min. The reactions were stopped by precipitation with mercuric chloride.[5] Inorganic nitrite formation, which indicated NG degradation, was determined by measuring the color complex formed in the presence of procaine hydrochloride (0·3% w/v) and N-(1-naphthyl)-ethylenediamine dihydrochloride (0·1% w/v).[6] Protein concentration was determined by the biuret procedure. Specific activity was expressed as micromoles of nitrite per ml per 10 min per mg protein.

Chromatographic separation of organic nitrates

Mixtures of organic nitrates were separated by thin-layer chromatography; 0·25-mm silica Gel G (Brinkmann) layers were used, which were activated at 110° for 30 min. The organic nitrates were extracted from aqueous incubations with diethyl ether and were concentrated by vacuum evaporation. After sample applications, the chromatogram was developed in 30 min by the ascending technique, with a solvent mixture of benzene and ethyl acetate in a 4:1 v/v ratio. Color development was achieved by spraying with diphenylamine (1% in alcohol), drying, and exposing the plates to short-wave u.v. light for 10 min.[7] Permanent records were made on positive blue printing paper (Driprint HC 241 B, Eugene Dietzgen Co., Washington, D.C.). Silica gel (100 mesh) columns were employed for the separation of large amounts of organic nitrates.

Coronary flow and blood pressure

Coronary flow was measured in the isolated beating hearts by a modified Langendorff technique.[8] Dog blood pressure was measured indirectly on a continuous systolic monitor (Beckman model SM-2).

RESULTS AND DISCUSSION

Drug metabolism is generally achieved by converting lipid-soluble compounds into more polar derivatives, which are more readily excreted by the kidney. The transformation of NG in the presence of GSH produces two organic nitratrate products which are less lipid soluble than is the parent molecule. Chromatographically, there were no differences in the NG metabolites produced by: (a) the reaction of NG and GSH in the absence of tissue; (b) the nonenzymatic reaction between NG and GSH in the presence of heart; (c) the liver enzyme-catalyzed reaction of NG and GSH (Fig. 1A); (d) the urinary excretion products following NG administration to adult rats (Fig. 1B); and (e) the synthetic 1,2- and 1,3-dinitroglycerins (Fig. 1B). The urinary metabolites (Fig. 1B) represent the pooled urine of three rats that were given 20 mg NG orally in 15 ml distilled water. The 6-hr urine contained some intact NG, larger amounts of the two dinitroglycerins, and a trace above the origin, which was probably a mononitrate.

After 24 hr, NG was no longer present in the urine and, by 30 hr, no nitrates could be found. The dinitrates appeared to be the major urinary metabolite after NG administration.

Mixed 1,2- and 1,3-dinitroglycerin (DNH) were prepared according to the method of Will.[9] The dinitrates, being more polar than nitroglycerin, can be separated from the parent molecule with water or polar solvents. The nitroglycerin metabolites and the synthetic dinitrates showed the same migration characteristics on thin-layer chromatograms, and their infrared spectra produced peaks of the same qualitative position and intensity. Thus, the major NG metabolites were analogous to 1,2- and 1,3-dinitroglycerin.

The production of the dinitrate metabolites of NG can be correlated with the formation of inorganic nitrite. Figure 2 represents the time course of generation of the metabolites of NG taken from aliquots of an incubation of NG and GSH in the presence of liver. The chromatogram shows a steady depletion of NG and generation of metabolites. The metabolites began to appear in low concentrations at 10 min, and the reaction continued to generate metabolites without evidence of the production of any other nitrated compounds. Intact NG was not completely metabolized in 24 hr. The production of inorganic nitrite followed a similar pattern with only low levels of nitrite in the early time periods. Nitrite production continued throughout the incubation period, because NG was still present. Since 1,2- and 1,3-dinitroglycerin appear to be the major metabolites, the nitrite is probably released from either the 2- or 3-position of NG. No need for further transformation seems necessary, since the dinitrates are the primary organic nitrates appearing in the urine.

Preliminary investigations were performed on the limited supply of dinitroglycerin available. A study of the metabolism *in vitro* revealed that the spontaneous reaction between dinitroglycerin and GSH caused no nitrite production and that the level exhibited in the presence of liver was insignificant as compared with NG (Table 1).

TABLE 1. COMPARISON OF THE *IN-VITRO* METABOLISM OF DINITROGLYCERIN TO NITROGLYCERIN*

Treatment	(μmoles nitrite/ml/10 min)			
	Alone	GSH	Liver	GSH + Liver
DNG	0·00	0·00	0·10	0·23
NG	0·00	0·16	0·19	2·03

* Concentrations employed per ml: dinitroglycerin (DNG) (synthetic mixture) 4 μmoles; GSH 8 μmoles; cyanide 8 μmoles; liver, 0·5 ml of the dialyzed 10,000-*g* supernatant of a 10% rat liver homogenate.

Thus, it appeared that dinitroglycerin was relatively resistant to further metabolism, which correlates with the chromatographic evidence in Fig. 2, and which lends support to the finding of Hunter and Ford[3] that not more than one nitrite is released per molecule of NG.

Also, studies of the pharmacologic activity of dinitroglycerin were performed. Coronary flow was measured in the isolated beating hearts of four rabbits. Nitroglycerin appeared to be more active as a coronary vasodilator and caused a greater depression of the amplitude of myocardial contraction than did the dinitroglycerins.

A comparison was made at a dosage of 1·0 mg, which showed that NG caused an average increase in coronary flow of 11·1 ± 2·0%, whereas the mixture of dinitroglycerin caused a 2·7 ± 1·2% increase.

The blood pressures of two dogs were measured indirectly on a continuous systolic monitor, and NG was found to be markedly more potent as a systolic blood pressure depressing agent than the dinitroglycerins. At a dosage of 1·0 mg, NG caused an average fall of systolic pressure of 12%, while the dinitroglycerin mixture caused an 11% fall at a dosage of 8·0 mg. The therapeutic activity of NG does not appear to reside in the dinitroglycerins.

The dinitroglycerins were found to be relatively resistant to further transformation and were isolated as the urinary excretion products after the administration of NG. The oil-water coefficient of organic nitrates appeared to be a determining factor in the potency of vasodilator nitrates.[10] The dinitroglycerins were more water soluble than NG[11] and were found to be less active pharmacologically as coronary vasodilators and as blood pressure depressing agents. Therefore, NG appeared to exert its activity by means of the intact molecule rather than by an active metabolite in the form of dinitroglycerin.

An investigation of several properties of the NG-metabolizing enzyme has been useful in characterizing the transformation reaction. Rat liver homogenates were employed to investigate the subcellular localization of the NG-metabolizing enzyme system. The apparent distribution of the enzyme is indicated in Table 2. The primary

TABLE 2. ENZYMATIC METABOLISM OF NITROGLYCERIN BY DIFFERENTIALLY CENTRIFUGED FRACTIONS OF RAT LIVER HOMOGENATE (5%)*

Fraction	Activity	
	NG	NG + GSH
	(μmoles nitrite/ml/10 min)	
Homogenate	0·20	1·08
600 *g*, S	0·25	1·39
10,000 *g*, S	0·12	1·24
10,000 *g*, P	0·00	0·26
100,000 *g*, S	0·12	1·08
1000,000 *g*, P	0·00	0·26

* Liver homogenates were prepared in 0·25 M sucrose in 0·01 M Tris buffer (pH 7·4). S = supernatant; P = pellet. All values represent the average of replicate experiments which have been corrected for the nontissue control of either NG or NG + GSH.

site of the NG-metabolizing enzyme appears to be in the 100,000-*g* soluble fraction rather than in the mitochondrial pellet as previously reported.[3] The low level of mitochondrial activity was probably attributable to contamination with soluble fraction. Nitrite-forming activity normally present in the supernatant fractions can be attributed to endogenous GSH levels, but the addition of exogenous GSH increased activity. There was no redistribution of the NG-metabolizing enzyme when the fractions were prepared in hypertonic sucrose (0·88 M) media.

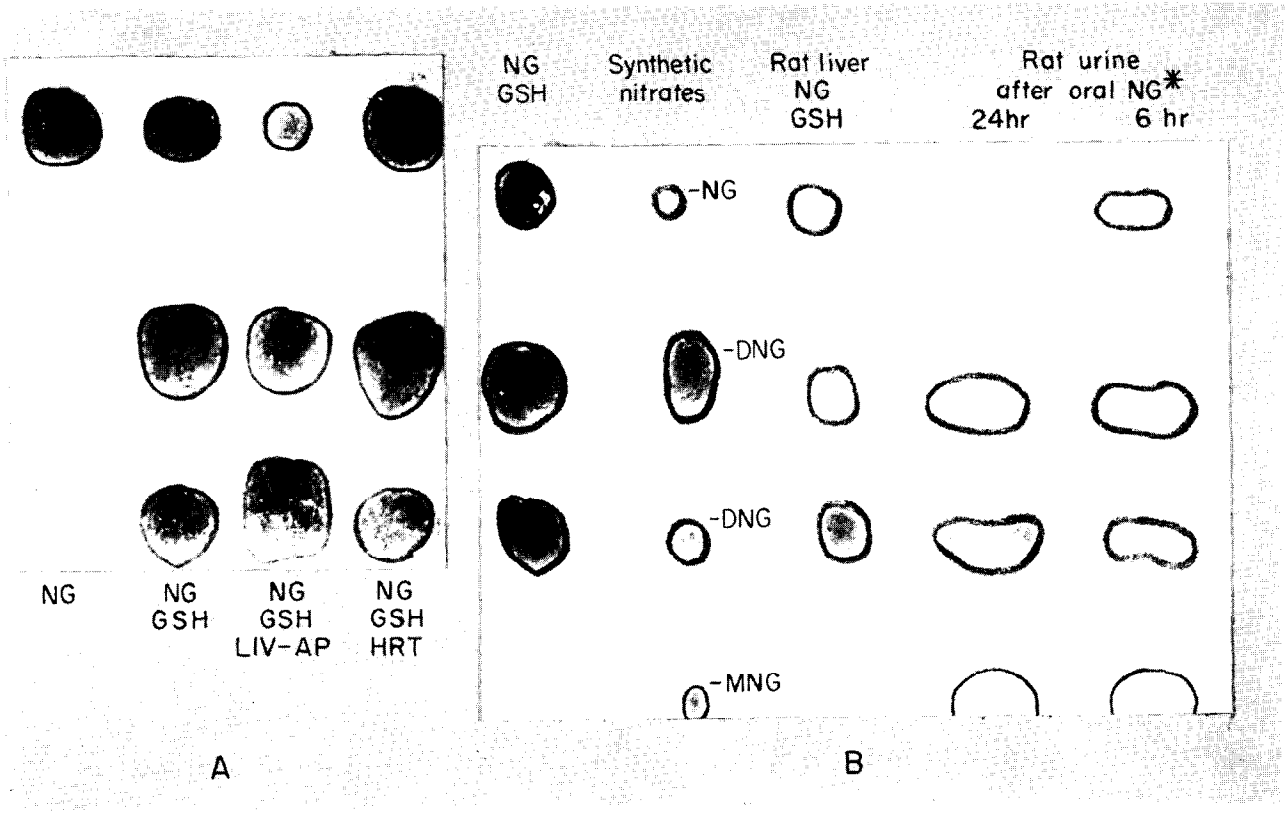

FIG. 1. A. Chromatographic results of incubating nitroglycerin (NG) with glutathione (GSH) in the presence or absence of tissue. Liv-AP = liver acetone powder; HRT = heart homogenate.

B. Comparison of the urinary, enzymatic, and nonenzymatic metabolites of nitroglycerin to the synthetic organic nitrates. DNG upper = 1,2-dinitroglycerin; DNG lower = 1,3-dinitroglycerin; MNG = mononitrates.

* NG, 20 mg p.o. in 15 ml HOH.

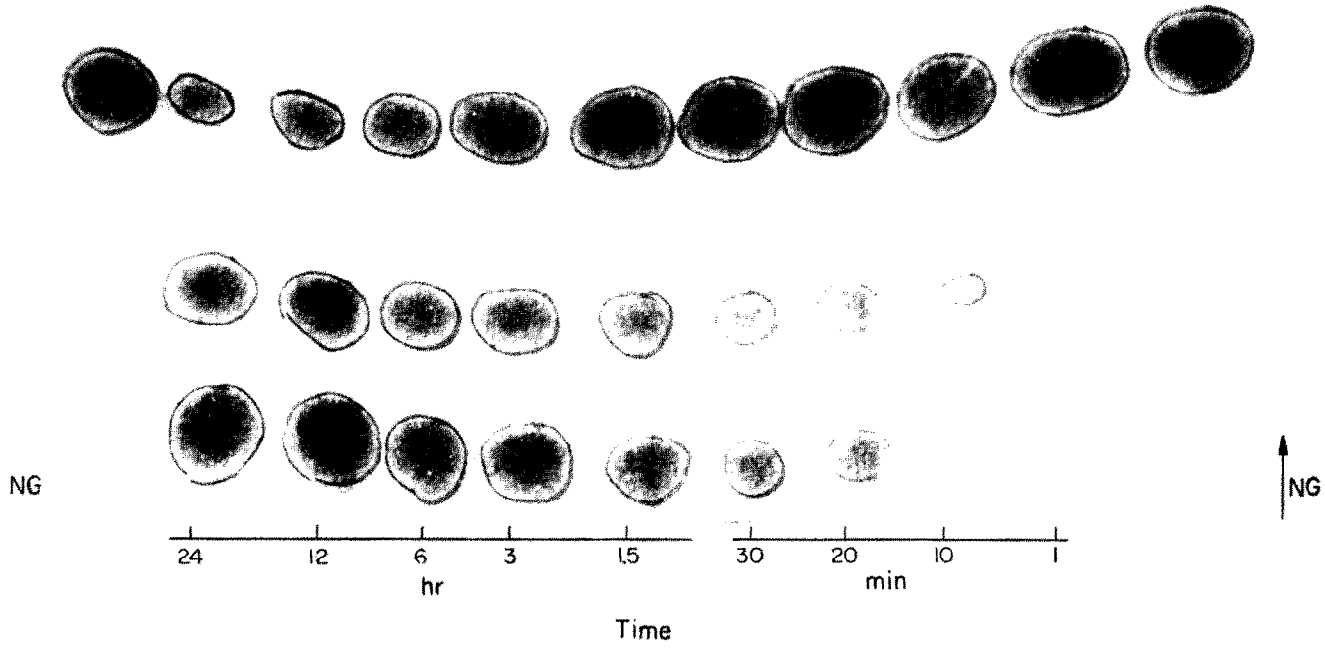

FIG. 2. Chromatogram of the time course of appearance of the metabolites of NG. Time preparations contained: 4 μmoles NG/ml, 8 μmoles GSH/ml, 8 μmoles cyanide/ml, 0·5 mg liver acetone powder (7%) per ml. Controls contained no GSH or tissue.

Figure 3 represents the effect of continuous-flowing dialysis on various acetone powder preparations incubated with a fixed amount of NG–GSH. The following results were observed: (a) the undialyzed preparations, with the exception of unheated liver, were less active than the control; (b) dialysis increased the activity of all preparations; and (c) only liver showed a greater activity than the control. Equilibrium dialysis

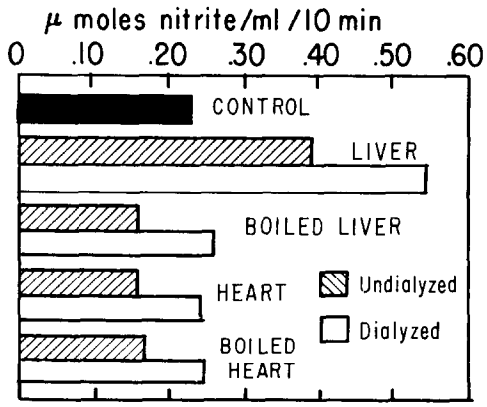

FIG. 3. The effect of continuous-flowing dialysis on the metabolism of nitroglycerin by heart and liver acetone powder preparations. Dialysis was against phosphate buffer (0·067 M, pH 7·4) flowing overnight. All preparations contain 4 μmoles nitroglycerin/ml and 8 μmoles glutathione/ml; the control contains no tissue.

indicated that the heat-stable inhibitor was present in the dialysate, which probably caused the oxidation of the GSH before its reaction with NG. Such sulfhydryl oxidation could be reduced by cyanide, which cleaves disulfide bonds.[12] The disulfide cleavage would explain the observation of Heppel and Hilmoe[2] that cyanide produced a 35% increase in nitrite-forming activity. Dialysis did not increase the NG-metabolizing activity of the heart above the level of the nontissue control, and it therefore appears that NG is not enzymatically metabolized to inorganic nitrite by the heart.

Phenobarbital pretreatment of adult rats for four days was found to enhance significantly ($P < 0{\cdot}05$) the biotransformation of NG. The homogenates obtained from the livers of saline-injected rats metabolized NG at a rate of $1{\cdot}71 \pm 0{\cdot}10$ μmoles/ml per 10 min per mg protein (corrected for NG–GSH nontissue control), whereas phenobarbital-pretreated livers had a rate of $2{\cdot}11 \pm 0{\cdot}12$ μmoles/ml per 10 min per mg protein. The activity of phenobarbital to enhance drug metabolism is generally attributed to enhancement of the activity of the liver microsomal enzymes which metabolize foreign compounds.[13, 14]

The enhancement of soluble NG-metabolizing liver enzyme by phenobarbital represents an unusual effect. Bresnick and Lin[15] found that phenobarbital pretreatment caused a twofold increase in the activity of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in rat liver. The resultant increase in NADPH in the presence of glutathione reductase would tend to increase the levels of GSH, a factor that might explain the enhanced NG metabolism that was induced by phenobarbital.

The ability of the NG-metabolizing enzyme to respond to dialysis, cyanide, and phenobarbital pretreatment indicates that it differs from: (a) the enzymes that form GSH conjugates with aromatic and aliphatic halogen and nitro compounds which are inhibited by dialysis or phenobarbital pretreatment;[16, 17] and (b) hog liver nitroreductase which is inactivated by cyanide.[18]

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