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NITROGLYCERIN BIOTRANSFORMATION BY RAT BLOOD SERUM

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Abstract—The degradation of ^{14}C-labeled glyceryl trinitrate by rat blood serum was investigated. Attention was focused upon the first de-esterification which yielded inorganic nitrite and the two isomeric glyceryl dinitrates. Cleavage of the nitrate group at carbon-2 was favored 4-fold over attack upon each terminal nitrate. The conversion proceeded optimally at approximately pH 7·8 and within the temperature range of 50–57°. Iodoacetamide and *p*-chloromercuribenzoate inhibited the ability of rat serum to transform nitroglycerin. Serum activity was not lost by dialysis. The data are interpreted as indicating that the de-esterification of nitroglycerin by serum is enzymatic and involves the reduction of organic nitrate to organic nitrite followed by the hydrolysis of nitrite ester to inorganic nitrite. The V_{max} of the unpurified rat serum showed it to be more potent than the hog liver organic nitrate reductase described previously. Unlike the hog liver enzyme, the serum reductase does not require reduced glutathione. Lacking this requirement, the serum system is also free of dependence upon TPNH for the function of glutathione reductase.

NITROGLYCERIN, generally regarded as the most effective anti-anginal agent, is known to be absorbed extremely rapidly upon sublingual administration to man. Our interest in the early biochemical events which follow the absorption of nitroglycerin led us to investigate the biotransformation of the drug by serum. This study, performed with rat blood serum and ^{14}C-labeled nitroglycerin, employed methodology developed previously for the qualitative and quantitative assay of the drug and its metabolites.[1]

MATERIALS AND METHODS

^{14}C-nitroglycerin. Labeled nitroglycerin was synthesized by Lawrie's method[2] from (1,3-^{14}C)-glycerol and purified as described by Dunstan *et al.*[3] The product was 99·9 per cent radiochemically pure. For safe handling, the nitroglycerin was mixed with 19 parts by weight of chemically pure lactose. The specific activity of the mixture was 0·18 mc/g.

Thin-layer chromatography (TLC). Chromatograms were developed by the ascending technique on 2 × 8 in. plates coated with 250 μ silica gel G bound with calcium sulfate. The solvent consisted of benzene:ethyl acetate:acetic acid (16:4:1, v/v) and gave R_f values of approximately 0·00 for glycerol, 0·10 for glyceryl mononitrates, 0·30 for glyceryl-1,2-dinitrate, 0·45 for glyceryl-1,3-dinitrate and 0·60 for nitroglycerin.

Nitroglycerin degradation by acid. A solution of 10 mg of the ^{14}C-nitroglycerin–lactose mixture in 5 ml of 4 N HCl was kept at 37° for 5½ hr. Then a portion was removed, neutralized with cold 5 N NH_4OH, and immediately assayed by TLC. A radioscan of the chromatogram is shown in Fig. 1A. The reaction mixture consisted of 76% nitroglycerin, 16% glyceryl-1,2-dinitrate and 8% glyceryl-1,3-dinitrate.

Nitroglycerin degradation by serum. A mixture of 3·0 ml of fresh rat serum and 3·0 ml of 0·2 M phosphate buffer (pH 7·8) was brought to 37° by mechanical agitation in a water bath. The system was made 5×10^{-5} M with respect to the substrate by adding 72 μg ^{14}C-nitroglycerin in 0·6 ml of distilled water, and the shaking at 37° was then resumed. Aliquots (0·5 ml) of the reaction mixture were removed after 1, 2, 5, 10, 30 and 60 min. Each sample was transferred immediately to 2·0 ml of ice-cold methanol to stop the reaction. The procedure used to collect all (>98·5%) of the organic nitrates from each mixture involved: (a) centrifugation and decanting of the supernatant liquid; (b) suspension of the precipitate in 1·0 ml of fresh cold methanol, centrifugation and decanting of the free liquid; and (c) rinsing the inner walls of the centrifuge tube with about 0·5 ml methanol. The combined supernatants and rinse liquid were often too turbid for direct application to thin-layer plates. In order to remove insoluble material without loss of organic nitrates, the mixtures were centrifuged after adding 2·0 ml of cold methanol. Then each supernatant was concentrated for TLC by evaporation to 100–300 μl in an air current at room temperature. Chromatograms were developed after spotting 100-μl aliquots on silica gel plates.

Effect of pH upon enzyme activity. A series of 0·2 M phosphate buffers was prepared; solutions with pH values outside of the usual range were prepared by adding HCl or NaOH without changing the molarity of the phosphate. One ml of rat serum was added to 1·0 ml of each phosphate solution and the pH of each mixture was read. Then 8 μg ^{14}C-nitroglycerin in 0·2 ml of distilled water was added to the buffered sera. The mixtures were incubated with shaking at 37° for 1 hr and assayed by TLC as described above. Control conversions were run over the same pH range by substituting water for serum.

Effect of temperature upon enzyme activity. Each enzymatic conversion was run for 30 min by shaking a mixture of 0·5 ml of rat serum, 0·5 ml of 0·1 M phosphate buffer at pH 7·8 and 40 μg ^{14}C-nitroglycerin in 0·2 ml water. Control tubes, containing buffer in place of serum, were run simultaneously. The temperatures investigated were 25°, 30°, 37°, 45°, 55°, 60° and 73°. The procedure for stopping the reaction and preparing solutions for TLC were the same as those described above, except that the final concentrations were carried out only to 0·9 to 1·0 ml.

Comparison of the molar proportions of nitrite ion and glyceryl dinitrates formed by nitroglycerin metabolism. Four different preparations of fresh rat serum were used to determine the relative quantities of inorganic nitrite and glyceryl dinitrates produced from nitroglycerin. In each experiment, a mixture of 0·5 ml serum and 0·5 ml of 0·1 M phosphate buffer at pH 7·8 was brought to 50° before adding ^{14}C-nitroglycerin solution to substrate levels of $2{\cdot}5 \times 10^{-4}$ M or $5{\cdot}0 \times 10^{-4}$ M. Then the mixtures were shaken mechanically in a water bath at 50° and the samples were removed after 15, 30 and 60 min. One aliquot (0·2 ml) was transferred directly to a tube containing 1 ml of ice-cold methanol and assayed for glyceryl nitrates as described above. The other aliquot (0·1 ml) was mixed with 3 ml of ice-cold trichloroacetic acid (5%) and centrifuged to remove protein. The supernatant solution was assayed for nitrite ion by adding 0·1 ml of 1% sulfanilamide in 0·05 N HCl, 0·1 ml of 0·5% *N*-(1-naphthyl)ethylenediamine in 1% HCl and 1 drop of concentrated HCl.[4] After the mixture was diluted to 5·0 ml with distilled water and allowed to stand at room temperature for 20 min, the optical density at 522 mμ was read. The nitrite ion concentration was calculated from a standard curve obtained from sodium nitrite solutions containing trichloroacetic acid.

Reaction of nitrite with serum. $NaNO_2$ solutions were prepared at concentrations of 7·5, 15, 30, 60 and 120 μg per ml of 0·1 M phosphate buffer at pH 7·8. A 0·5-ml aliquot of each solution was mixed with an equal volume of fresh rat serum. Immediately, two 0·1-ml aliquots were withdrawn from the serum mixture, added to 3·0 ml of ice-cold 5% trichloroacetic acid solution and centrifuged. The remainder of the serum mixture was incubated at 50°. Aliquots were removed after 15 and 60 min, transferred directly to trichloroacetic acid solution, and centrifuged as above. The supernatant solutions and controls consisting of $NaNO_2$, buffer and trichloroacetic acid were assayed for nitrite.[4]

Effect of rat blood serum upon inorganic nitrate. To 0·5 ml of fresh serum was added 0·5 ml of a solution containing 42·5 μg $NaNO_3$ per ml of phosphate buffer (0·1 M, pH 7·8). Aliquots for nitrite assay[4] were withdrawn immediately and after incubation at 50° for 15, 30 and 60 min. No nitrite was detected. The experiment was repeated with the same result.

Effect of dialysis upon activity of serum enzyme. A section of dialysis tubing containing 1·0 ml of rat serum was immersed in 20 ml of 0·2 M phosphate buffer (pH 7·8) for 30 min and then transferred to fresh buffer. This process was performed 6 times and followed by dialysis against fresh buffer for 18 hr. The dialyzed serum and untreated serum were assayed for ability to metabolize nitroglycerin in a system consisting of 0·1 ml serum and 1·0 ml of phosphate buffer containing 8 μg ^{14}C-nitroglycerin. The incubations were carried out simultaneously for 60 min with shaking at 37°. After employing the usual procedure to determine the levels of the organic nitrates present, it was found that dialyzed serum converted as much nitroglycerin (55 per cent) to glyceryl dinitrates as did fresh serum.

Effect of reduced glutathione and TPNH on nitroglycerin degradation in the presence and absence of enzyme. Aliquots (0·6 ml) of a solution containing ^{14}C-nitroglycerin (40 μg) in 0·07 M phosphate buffer (pH 7·8) were added to: (a) 0·6 ml water containing 54 μg of reduced glutathione; (b) 0·5 ml of fresh serum plus 0·1 ml water; and (c) 0·5 ml serum plus 0·1 ml water containing 54 μg of reduced glutathione. The mixtures were incubated with agitation at 37°. Aliquots withdrawn after 15, 30 and 60 min were assayed in the usual manner. The entire experiment was repeated with 10 times as much reduced glutathione.

The same protocol was followed to observe (a) the effect of 10 molar equivalents of TPNH and (b) the combined effect of reduced glutathione and TPNH upon nitroglycerin de-esterification.

Enzyme inhibitors. The enzyme inhibitors tested were iodoacetamide, silver nitrate, *p*-chloromercuribenzoic acid, sodium azide and oxalic acid at final concentrations of 10^{-3}, 10^{-4} and 10^{-5} M. Rat serum was diluted with 2 vol. of 0·1 M phosphate buffer (pH 7·8). Two-ml aliquots of the buffered serum were mixed with 0·3 ml of each inhibitor solution and 0·2 ml ^{14}C-nitroglycerin solution (200 μg/ml). The mixtures were incubated with shaking for 1 hr at 37° and then assayed for glyceryl nitrates by the usual procedure. Controls were run with water replacing the inhibitor solution.

Determination of Michaelis constant and maximal velocity. Three solutions with different concentrations of ^{14}C-nitroglycerin were prepared in phosphate buffer (0·2 M, pH 7·8). These substrate solutions and rat serum were heated separately to 37°. Then 1·0 ml serum was added to 2·0 ml of each substrate solution; the final nitroglycerin concentrations were 0·25, 0·5 and 1·0 mM. The mixtures were shaken at

37° and 0·5-ml aliquots were withdrawn after 1, 2, 5 and 10 min. The conversions were stopped with methanol, and the organic nitrates were extracted and assayed as described above. Control runs were made with buffer in lieu of serum.

RESULTS

Figure 1A shows a radioscan of a chromatogram developed after the acid treatment of ^{14}C-nitroglycerin. Twice as much glyceryl-1,2-dinitrate as glyceryl-1,3-dinitrate was produced. This result was expected because the terminal nitrate groups of nitroglycerin are identical. The situation is quite different in Fig. 1B, a radioscan reflecting the

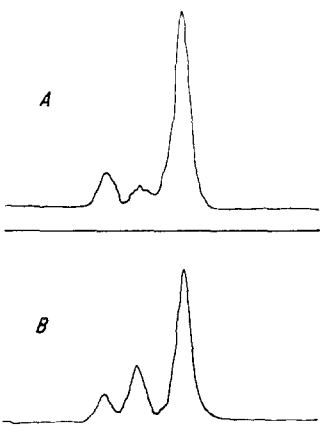

FIG. 1. Radioscans of ^{14}C-nitroglycerin degradation by (A) acid and (B) rat blood serum. The peaks represent glyceryl-1,2-dinitrate (R_f 0·32), glyceryl-1,3-dinitrate (R_f 0·43) and nitroglycerin (R_f 0·57).

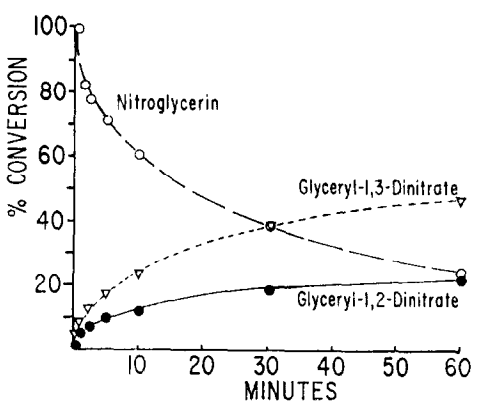

FIG. 2. Time course of nitroglycerin biotransformation by rat blood serum.

status of the biotransformation of ^{14}C-nitroglycerin by rat blood serum after incubation for 10 min. It is evident from this illustration that the proportion of glyceryl-1,3-dinitrate to glyceryl-1,2-dinitrate was approximately 2:1. Figure 2 depicts the course of the conversion of nitroglycerin to the glyceryl dinitrates. The 2-nitrate group was cleaved preferentially throughout the 1-hr study period over which 76 per cent of the nitroglycerin was degraded.

Figure 3 was constructed by subtracting the control from the serum conversion values and shows the enzyme system to have peak activity in the region of pH 7·8. The enzyme was virtually inactive below pH 6 and above pH 10·5.

After correcting the data for purely thermal effects, the activity of the serum enzyme toward nitroglycerin was found to be maximal in the range of 50–57° (Fig. 4). At body temperature, the enzyme showed about 70 per cent of its peak activity.

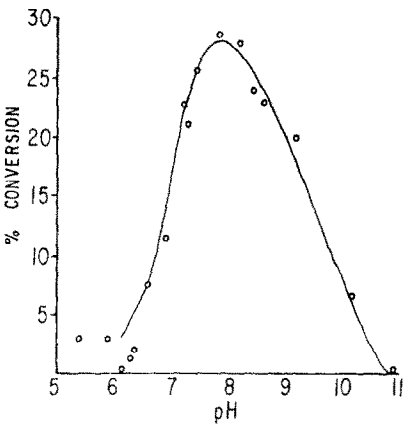

FIG. 3. Effect of pH on the biotransformation of nitroglycerin by rat blood serum; 100% conversion would represent the complete de-esterification of all of the nitroglycerin to glycerol.

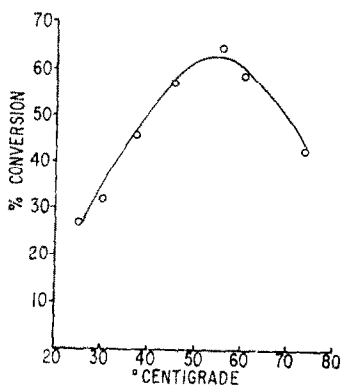

FIG. 4. Effect of temperature on nitroglycerin biotransformation to glyceryl dinitrates by rat blood serum.

The data in Table 1 show that inorganic nitrite was not found in levels equivalent to those of glyceryl dinitrates. In the four experiments, the mean molar ratios of nitrite ion to glyceryl dinitrates were 0·33, 0·67, 0·36 and 0·73. These proportions did not depend upon the substrate concentration; one high and one low value were obtained at each of the two initial nitroglycerin levels.

Table 2 shows that inorganic nitrite was lost by mixing with serum. The quantities recovered did not vary with the incubation time and the recovery was approximately 50 per cent regardless of the initial $NaNO_2$ concentration.

TABLE 1. NITRITE ION AND GLYCERYL DINITRATES FOUND AFTER INCUBATING ^{14}C-NITROGLYCERIN WITH SERUM

Serum	Incubation (min)	Nitroglycerin (M)	% Conversion to		Molar ratio NO_2^-/glyceryl dinitrates
			Nitrite*	Glyceryl dinitrates	
A	15	$2{\cdot}5 \times 10^{-4}$	13	51	0·25
	30		21	71	0·30
	60		34	76	0·45
B	15	$2{\cdot}5 \times 10^{-4}$	31	50	0·62
	30		42	62	0·68
	60		50	70	0·71
C	15	$5{\cdot}0 \times 10^{-4}$	10	35	0·29
	30		16	40	0·40
	60		17	44	0·39
D	15	$5{\cdot}0 \times 10^{-4}$	32	44	0·73
	30		36	48	0·75
	60		35	49	0·71

* The nitrite values relate to the removal of a single nitrate from nitroglycerin and are comparable directly to per cent conversion to glyceryl dinitrates.

TABLE 2. RECOVERY OF NITRITE ION FROM MIXTURES OF $NaNO_2$ AND RAT BLOOD SERUM

$NaNO_2$ (μg/ml)	Incubation time (min)	% $NaNO_2$ remaining
60	0	56·7
60	15	55·0
60	60	55·0
30	0	56·6
30	15	56·6
30	60	56·6
15	0	42·0
15	15	51·6
15	60	54·7
7·5	0	42·2
7·5	15	47·7
7·5	60	52·7
3·75	0	46·0
3·75	15	49·1
3·75	60	49·1

TABLE 3. EFFECT OF GLUTATHIONE AND TPNH ON NITROGLYCERIN DEGRADATION IN THE PRESENCE AND ABSENCE OF SERUM

Serum (ml)	NG (μM)	GSH (μM)	TPNH (μM)	% Conversion to glyceryl dinitrates		
				15 min	30 min	60 min
	0·15	0·15		0	0	0
A 0·5	0·15			28	37	52
A 0·5	0·15	0·15		36	45	56
	0·15	1·5		9	13	22
B 0·5	0·15			26	36	51
B 0·5	0·15	1·5		45	58	68
	0·15		1·5	0	0	0
C 0·5	0·15			41	51	64
C 0·5	0·15		1·5	40	45	61
	0·15	0·15	0·15	0	0	0
D 0·5	0·15			32	49	54
D 0·5	0·15	0·15	0·15	37	57	67

It is evident from Table 3 that glutathione reacted chemically with nitroglycerin, but that glyceryl dinitrates were not formed in appreciable quantities unless a large excess of glutathione was used. On the other hand, 10 molar equivalents of TPNH failed to de-esterify nitroglycerin. The presence of glutathione or TPNH or of both in incubation mixtures failed to accelerate the transformation of nitroglycerin by serum beyond the strictly chemical effect of glutathione.

Effective inhibitors of the serum enzyme were iodoacetamide, silver nitrate and *p*-chloromercuribenzoate (Table 4), indicating that the enzyme contains sulfhydryl groups essential for its activity. The failure of sodium azide to inhibit the enzyme indicates that it is not a ferric protoporphyrin, and the lack of effect of oxalate shows that calcium and magnesium ions are not necessary for the enzyme to express its activity.

TABLE 4. EFFECT OF INHIBITORY AGENTS UPON NITROGLYCERIN TRANSFORMATION BY RAT BLOOD SERUM

Inhibitor	Molarity	% Conversion to glyceryl dinitrates	% Inhibition
		70	
Iodoacetamide	10^{-3}	0	100
Iodoacetamide	10^{-4}	6	90
Iodoacetamide	10^{-5}	64	0
Silver nitrate	10^{-3}	15	80
Silver nitrate	10^{-4}	25	65
Silver nitrate	10^{-5}	57	20
p-Chloromercuribenzoate	10^{-3}	19	70
p-Chloromercuribenzoate	10^{-4}	47	30
p-Chloromercuribenzoate	10^{-5}	56	20
Sodium azide	10^{-3}	69	0
Sodium azide	10^{-4}	77	0
Sodium azide	10^{-5}	72	0
Oxalic acid	10^{-3}	71	0
Oxalic acid	10^{-4}	67	0
Oxalic acid	10^{-5}	63	0

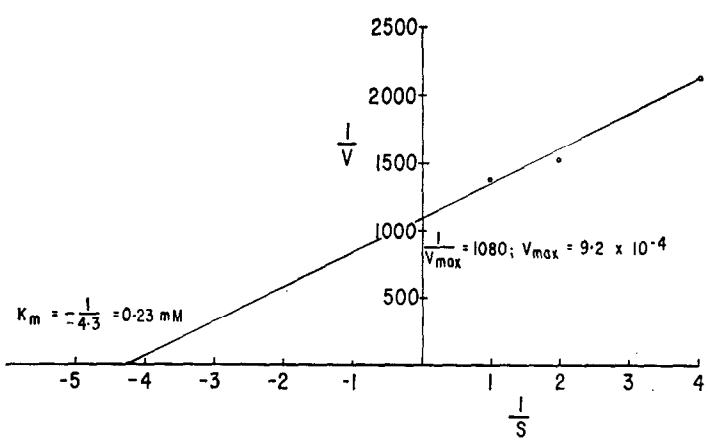

FIG. 5. Lineweaver–Burk plot of 1/V versus 1/S for nitroglycerin biotransformation by rat blood serum; $V_{max} = 9{\cdot}2 \times 10^{-4}$ m-mole of glyceryl dinitrates formed/mg of protein/min. The Michaelis constant, K_m, determined by extrapolation to the abscissa, equals 0·23 mM. K_m represents the substrate concentration giving half-maximal velocity.

Figure 5 shows a Lineweaver–Burk[5] plot of the biotransformation of nitroglycerin in various concentrations by the same quantity of serum. From the ordinate intercept, V_{max} was found to be $9{\cdot}2 \times 10^{-4}$ ($\pm$ $0{\cdot}8 \times 10^{-4}$) m-mole of glyceryl dinitrates formed per mg of serum protein per min. Extrapolating the line to the abscissa[6] gave $K_m = 2{\cdot}3 \times 10^{-4}$ ($\pm$ $0{\cdot}9 \times 10^{-4}$) M.

DISCUSSION

Earlier studies showed that nitroglycerin administered to rats is converted to glyceryl nitrates, glycerol, organic acids, carbon dioxide[7, 8] and to tissue components including protein, lipid, glycogen, RNA and DNA.[9] The first step in the biotransformation of nitroglycerin yields the isomeric dinitrates of glycerol.[1, 10, 11] These dinitrates are produced *in vitro* by the action of liver[11, 12] and blood plasma[13] enzymes upon nitroglycerin. The same enzymes de-esterify other organic nitrates.[11, 14]

Needleman and Hunter[11] studied the hog liver organic nitrate reductase in a system containing added TPNH, reduced glutathione and glutathione reductase. After correlating the extent of nitroglycerin conversion with the TPNH consumption, they proposed the following 3-step mechanism for the removal of a nitrate group:

$$2\ \text{TPNH} + \text{GSSG} \longrightarrow 2\ \text{TPN} + 2\ \text{GSH}$$
$$2\ \text{GSH} + \text{RONO}_2 \longrightarrow \text{GSSG} + \text{H}_2\text{O} + \text{RONO}$$
$$\text{RONO} + \text{H}_2\text{O} \longrightarrow \text{ROH} + \text{HNO}_2$$
$$\overline{2\ \text{TPNH} + \text{RONO}_2 \longrightarrow 2\ \text{TPN} + \text{ROH} + \text{HNO}_2}$$

We found that serum requires neither TPNH nor reduced glutathione to degrade nitroglycerin. Excluding these compounds from the conversion mechanism, we considered two possible pathways for the de-esterification of nitroglycerin. One possible mechanism involved: (a) hydrolysis to release inorganic nitrate from nitroglycerin, and (b) the action of an inorganic nitrate reductase to produce the inorganic nitrite which is actually found. This pathway was rejected when we observed no nitrite formation after incubating inorganic nitrate with serum. Therefore, we consider that the biotransformation of nitroglycerin by serum proceeds by: (a) the reduction of organic nitrate to organic nitrite and (b) the hydrolysis of organic nitrite to ROH plus inorganic nitrite.

No one has synthesized either of the mononitrites produced by the reduction of one nitrate group in nitroglycerin. In fact, the only glyceryl nitrite described in the literature is glyceryl trinitrite[15]. This compound is a liquid which reacts very quickly with water. If the glyceryl dinitrate mononitrites are also highly sensitive to hydrolysis, there is no need for an enzyme. Assuming that the hydrolytic step proceeds simultaneously, we may focus our attention upon the first step in the mechanism, i.e. the reduction of nitroglycerin. It is conceivable that this reaction is also purely chemical. The reductant may be a nondialyzable or strongly bound sulfhydryl compound present in serum. Increasing the temperature of the serum–nitroglycerin reaction mixture, however, did not accelerate the conversion rate so greatly as expected of a chemical reaction. In addition, the optimum temperature range for the reaction suggests the involvement of a protein. Extremely difficult to explain on a chemical basis is the pH optimum observed for the reduction of nitroglycerin by serum. There seems to be no report on a strictly comparable system, but Klotz *et al.*[16] found no significant change in the rate of reaction between protein sulfhydryl groups and an organic disulfide over the pH range

from 2·5 to 9·2. Therefore, it is our view that the reduction of nitroglycerin by rat serum is an enzymatic process. We also believe that the serum organic nitrate reductase is sufficiently characterized to clearly distinguish it from the glutathione-requiring liver reductase.

Since the lower glyceryl nitrates have less pharmacological activity than nitroglycerin,[17] the transformation of nitroglycerin by serum contributes to understanding the short duration of action of the drug. The metabolism of nitroglycerin by serum also has significant implications, because it is generally administered sublingually and enters the coronary circulation before it reaches the liver.

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