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REVIEW ARTICLE

Aspects of Nitrate Ester Metabolism

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Nitrate esters are those compounds formed by the nitration of alcohols and contain the chemical grouping —C—O—NO_2, with the nitrogen linked to the carbon atom *via* an oxygen atom. The best known ester is glycerol trinitrate (I)[1], which is representative of nitrates of polyhydric alcohols widely used in medicine and as explosives since the mid-nineteenth century. In medicine, they are used as drugs in the treatment of angina pectoris; in this context, they are still of primary importance, even though it is now over 100 years since Brunton described their pharmacological effects (1). The principal uses of nitrate esters in the explosives industry are in the manufacture of dynamite, which contains I as one of its main ingredients together with ethylene glycol dinitrate (II) when antifreeze properties are required. Personnel engaged in the manufacture of these explosives can be exposed to the vasodilator actions of the esters which can enter the body *via* the lungs or skin.

In view of the widespread therapeutic use of nitrate esters and the hazards arising from occupational exposure, they have been subjected to detailed pharmacological investigations in man and in animals. Several reviews on this subject appeared in recent years (2–9). Although their metabolism has been studied for nearly 100 years, no review has been devoted entirely to the subject. Two reviews (10, 11) in the mid-1940's included some discussion of metabolism as it affected toxicity and pharmacology.

Studies of the metabolism of nitrate esters developed originally from the efforts of earlier investigators to establish a relationship between their breakdown *in vivo* and the physiological effects produced. Particular interest centered on the formation of inorganic nitrite, since the physiological responses from nitrate esters, such as the ability to lower blood pressure, are very similar to those of the nitrite ion. As early as 1883, Hay (12) showed that when I was incubated with cat blood for 30–40 min., the ester was completely broken down and inorganic nitrite formed. Although his analytical method for showing this result was rather crude, subsequent studies confirmed this basic action of blood and other tissues on nitrate esters. The metabolism in blood was shown to occur almost wholly in the red cell, since separated plasma showed no power of breakdown of I while the erythrocytes had a greater activity than whole blood (13). Pentaerythritol tetranitrate (III) and erythritol tetranitrate (IV) also liberate nitrite when incubated with blood, and they produce this entity in blood when administered orally to dogs (14). The rates of formation of nitrite differ markedly, however, being slowest with III and fastest with IV.

It is evident that inorganic nitrite produced from the breakdown of nitrate esters is itself metabolized, as was indicated by very early studies. Leech (15) and Cash and Dunstan (16) showed that after an injection of sodium nitrite into animals, a large proportion appeared as

[1] Roman numerals in parentheses refer to list of nitrate esters in Table I.

Table I—Nitrate Esters

I	Glycerol trinitrate
II	Ethylene glycol dinitrate
III	Pentaerythritol tetranitrate
IV	Erythritol tetranitrate
V	Mannitol hexanitrate
VI	1,2-Glycerol dinitrate
VII	1,3-Glycerol dinitrate
VIII	Glycerol mononitrate
IX	Pentaerythritol[a]
X	Pentaerythritol mononitrate
XI	Pentaerythritol dinitrate
XII	Pentaerythritol trinitrate
XIII	Ethylene glycol mononitrate
XIV	Propylene glycol 1,2-dinitrate
XV	Propylene glycol 2-mononitrate
XVI	Propylene glycol 1-mononitrate
XVII	Isosorbide dinitrate
XVIII	2-Isosorbide mononitrate
XIX	5-Isosorbide mononitrate

[a] Not a nitrate.

nitrate in the urine. Oxidation also takes place in animal tissue homogenates where the disappearance of added nitrite is balanced by the appearance of nitrate (17).

The first investigations concerned solely with the mode of breakdown of nitrate esters without reference to pharmacological action were carried out by Oberst and Snyder (18) in 1948. They measured the rate of breakdown of I and mannitol hexanitrate (V) in liver homogenates by following the nitrite produced with time. The reaction was shown to be dependent upon hydrogen-ion concentration, with maximum activity at pH 8.4. In later studies, Heppel and Hilmoe (19) found that I reacted with reduced glutathione to liberate inorganic nitrite and that the reaction was catalyzed by a hog liver enzyme. These investigations were the forerunners of the much more detailed studies of the reaction mechanisms and metabolism of nitrate esters that have taken place in recent years.

METABOLISM

Glycerol Trinitrate (I)—TLC analysis of ether extracts from the reaction of I in liver homogenates and from the urine of rats dosed with I demonstrated the formation of 1,2- and 1,3-glycerol dinitrates (VI and VII) (20). *In vivo*, the course of metabolism can be followed by analyzing at timed intervals the pooled urine from rats dosed with I. After 6 hr., I is present along with larger amounts of the two dinitrates and a trace of glycerol mononitrate (VIII). After 24 hr., I is almost absent and the dinitrates are the major metabolites; after 30 hr., excretion appears almost complete since none of the nitrates can be detected. The formation of the more water-soluble dinitrates from I is another example of a classical metabolic reaction whereby a relatively lipid-soluble compound is converted to more polar derivatives.

In glutathione–liver enzyme-catalyzed reactions, 90% of the nitrate groups enzymically degraded in the I molecule can be accounted for by the appearance of VI, VII, and inorganic nitrite. From these studies, it seemed that the metabolism of I did not proceed significantly beyond the removal of one nitrate group, since VIII was detected only in trace amounts and glycerol not at all. It was possible that since nitrate assays were used for the detection of metabolites on TLC plates, a completely denitrated product such as glycerol would be overlooked. In fact, more recent studies (21) showed that glycerol is a product of the *in vivo* breakdown of I. After oral administration to rats, the ester is quickly absorbed and rapidly degraded to carbon dioxide. Combined radiochemical and TLC methods demonstrate that over half the I is absorbed from the GI tract within 30 min. of dosing and that maximum tissue levels of ^{14}C are attained in the same time. Within 4 hr., approximately equal amounts (20%) of ^{14}C are exhaled as CO_2 in the expired air and appear as metabolites in the urine. No unchanged I is detected, and it is apparent that degradation to CO_2 must proceed *via* denitration to glycerol, the principal urinary metabolite.

The extraction of tissues reveals an accumulation of nonextractable material as the breakdown proceeds, and it appears that glycerol enters normal metabolic processes leading to the deposition of ^{14}C-glycogen and, possibly, lipids. This observation was followed up with studies on the incorporation of ^{14}C from ^{14}C-I into rat liver glycogen, lipid, protein, and RNA (22). These were assayed 1 hr. after ^{14}C-I administration, when nonextractable material was shown to be maximal. Levels of liver lipid, glycogen, protein, RNA, and DNA are unaffected, but radioactivity is present in all components. The results of this study indicate that glycerol produced from I metabolism enters a variety of normal anabolic processes.

Part of the I metabolism takes place in blood since incubation with rat serum denitrates 76% of the ester in 1 hr. (23). Twice as much VII is produced as VI, a finding that is the reverse of *in vivo* results in urine (21). The pH maximum for the reaction is 7.8, and at 37° the activity is only 70% of that in the optimum temperature range at 50–57°. In rat plasma, I is bound to protein to the extent of 60%, as is VI, while VII shows only 35% binding. Therefore, the binding could be attributable mainly to adjacent nitrate groups (24).

Pentaerythritol Tetranitrate (III)—Cass *et al.* (25) predicted that III would denitrate during metabolism to give products containing one to three nitrate groupings. This prediction was amply confirmed by the extensive studies of Di Carlo and his coworkers on the metabolism of this compound. They realized that many previous studies on the comparative pharmacology and metabolism of nitrate esters relied only upon the measurement of liberated inorganic nitrate or nitrite and that little attempt had been made to find what other metabolites were formed. Using ^{14}C-III and TLC and radioscanning (26), they separated and determined III and the metabolites pentaerythritol (IX), pentaerythritol mononitrate (X), pentaerythritol dinitrate (XI), and pentaerythritol trinitrate (XII) formed during reactions *in vitro* and *in vivo*. Compound III is denitrated in human blood *in vitro* (27) mainly to XI and XII, with small amounts of IX and X. The reaction takes place mainly in the red cells, with 62% III denitration in 1 hr., although some degradation occurs in plasma (18% in 1 hr.). The denitration occurs stepwise, with III being converted to the trinitrate faster than XI is formed from the latter. Compound III is strongly bound to plasma protein, and XII less so, while IX, X, and XI are not bound to any degree. The binding of III (85%) is much stronger than I (60%).

Single oral doses of 10 mg. III/kg. body weight were used in metabolic studies on mice and rats (28–30). Within 4 hr. of dosing, ^{14}C reaches the maximal level in blood, principally in the form of IX, X, and XI. Blood clearance is rapid due to excretion and uptake into organs, where ^{14}C levels are higher than in blood after 1 hr. Compounds IX and X are the major metabolites in the tissues, showing that degradation is further advanced than in blood. Excretion of breakdown products *via* the lungs is negligible, amounting to less than 0.3% of the total dose in both mice and rats. In the 24-hr. urine, 36% of the dose is excreted mainly as IX from mice, and 25–30% is excreted from rats in the form of IX and X in the ratio of 5:3. The urinary excretion after the initial 24 hr. is very slow, less than 60% of the dose being accounted for in 4 days from mice. The absorption and excretion of III in mice were compared directly with those of its parent alcohol IX. The former, as was shown, is absorbed slowly and is excreted as IX; the latter is absorbed quickly, and most of it is excreted unchanged within 24 hr.

In man (31) the urinary excretion of metabolites after oral administration of ^{14}C-III is similar to that found in the rat. Compounds IX and X are the principal metabolites, together with a small amount of XI. The overall recovery of ^{14}C in the 24-hr. urine is approximately 36% of the original dose. In a study of patients with a known history of coronary artery disease, it was shown that there was no difference compared with a control group in the metabolism of III regarding blood levels attained and amounts of metabolites excreted after oral doses of the ester.

An estimation of the rate of absorption of III after oral administration was made from studies where the radiolabeled ester was placed directly into the ligated stomach or intestines of starved rats (32). The biggest absorption overall takes place from the large intestine, 12.9% in 4 hr., and the smallest from the stomach, 3.7% in 4 hr. The faster rate of absorption from the large intestine is ascribed to the fact that III is readily degraded to XII by bacterial flora. Furthermore, since this ester is more water and lipid soluble than the highly insoluble III, it is able to pass more easily through the intestinal wall. Scarcely any hydrolysis of III occurs in the stomach or small intestine. Compound III is absorbed much more slowly from the gut than is I, which shows up to 100% absorption in 6 hr. (33).

The apparently greater absorption of XII indicated in these studies led to an interest in the possibility of its use as an antianginal agent in its own right. Some absorption and metabolic studies were carried out in the rat, dog, and man (34–36) in conjunction with its clinical evaluation. In all three species, the trinitrate is more rapidly absorbed than III. In the rat, the trinitrate behaves similarly to I, with 60% of the ^{14}C dose absorbed from the GI tract in 4 hr. compared with 28% after III administration. Dog and man show similarities, with a fast urinary excretion of the ^{14}C label after oral XII administration, reaching 88% in the dog and 91.5% in man after 48 hr., the principal metabolites being IX and X. These two metabolites are the main breakdown products in the rat, but some unchanged trinitrate is also found in urine. The slower rate of metabolism in the rat is indicated by the significant levels of XII present in blood 4 hr. after administration, whereas the unchanged ester is absent after 1 hr. in dog and in man.

Studies on the biotransformation of III in heart subcellular fractions (37) are especially relevant since this organ is associated with the pharmacological action of nitrate esters. Nuclear, mitochondrial, postmitochondrial, microsomal, and postmicrosomal fractions of rat heart are all active in degrading III. The tri- and dinitrates are the principal metabolites after an 18-hr. incubation of the fractions at 37°, with 10 mcg. ^{14}C-III, nicotinamide, and a $NADPH_2$-generating system in KCl phosphate buffer at pH 7.4. Similarly, incubation of III with homogenates of parenchymal or reticuloendothelial cells of mouse liver shows degradation of the ester by both fractions, although neither produces complete conversion by removal of all four nitrate groupings (38). It is claimed that the results of this study differ from other investigations which showed that the parenchymal and reticuloendothelial fractions play specific and quite different roles in metabolism of compounds by the liver.

Ethylene and Propylene Glycol Dinitrates (II and XIV)—The metabolism of these two compounds is very similar and may be reviewed together. Administration of II to rabbits leads to maximal concentrations in the blood 1–2 hr. after injection, while inorganic nitrate produced by metabolism reaches its maximum at 4–5 hr. (39). The surprising finding of this study is that inorganic nitrite could not be detected in the blood in contrast to its detection by nearly all other workers after *in vitro* and *in vivo* reactions of nitrate esters.

Clark and Litchfield (40) presented a comprehensive picture of the fate of II in the rat, with measurement of blood levels after injection, together with complementary excretion and *in vitro* studies. Compound II quickly disappears from the bloodstream, and inorganic nitrite and nitrate are formed. Ethylene glycol mononitrate (XIII) is also formed; it seems probable that in the first instance II breaks down to the mononitrate and inorganic nitrite, the latter being rapidly oxidized to nitrate, thus accounting for its own disappearance from the bloodstream and its absence from urine. Inorganic nitrate persists for a longer period in the bloodstream and, since it is stable, it is removed by the normal excretory processes. It does, in fact, form the major metabolite in the urine, accounting for nearly 60% of the original dose of II within 24 hr.

Compound XIII is also rapidly metabolized *in vivo;* after injection in rats, only a trace is excreted unchanged. During the metabolism, a small amount of inorganic nitrite is present in blood, presumably as an intermediate in the formation of inorganic nitrate which is again the major metabolite in urine. Ethylene glycol is a urinary metabolite from the *in vivo* breakdown of II and XIII, showing that complete denitration is achieved for both esters. Propylene glycol 1,2-dinitrate (XIV) is similarly metabolized in the rat (41) to give inorganic nitrite and nitrate in blood, with the latter a major metabolite in the urine. This ester, however, gives rise to two mononitrates due to partial denitration; since less than 0.5% of either is excreted in urine, it is assumed that they can be rapidly metabolized in the same way as

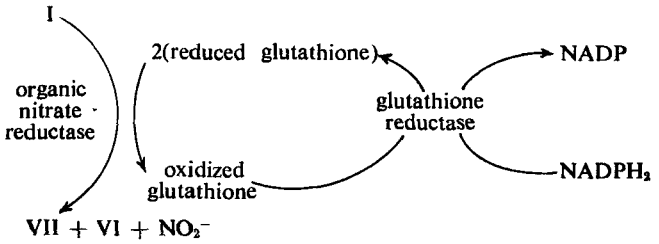
XIII. Propylene glycol 2-mononitrate (XV) predominates in the blood, and it seems likely that there is a reaction favoring the formation of this isomer.

The initial breakdown of the dinitrates to mononitrates and nitrite can be simulated in blood *in vitro*. The reaction is shown to occur entirely in the red cells, the plasma having minimal activity. Compound XIII is stable under these conditions, and it is possible to account for all the breakdown products of II *in vitro* after a 2.5-hr. incubation at 37°. Compound XIV gives rise to propylene glycol 1-mononitrate (XVI) and XV as well as inorganic nitrite. The 2-isomer again predominates as it does *in vivo;* and since both isomers are stable in blood *in vitro*, there must be a reaction favoring the formation of the former.

The metabolism of II and XIV in the rat is similar to that of I and III, since they denitrate to give mononitrates and a parent alcohol, and none of the original molecule is excreted unchanged in the urine. The fate of the carbon skeleton of II may be assessed by collation of the above results with those from the ^{14}C studies in mice (42). Excretion is rapid, 68% of the ^{14}C appearing in the first 24 hr., mainly in the urine and expired air. Volatile organic compounds account for only 2% of the activity in the expired air, the main breakdown product by this route, CO_2, amounting to 23% of the original activity. Compound II is, therefore, degraded *via* XIII to ethylene glycol, and the latter is then excreted partly unchanged in the urine and partly by complete breakdown to CO_2 in expired air. The terminal excretion and degradation of ethylene glycol is consistent with the observations of Gessner *et al.* (43).

Miscellaneous Nitrate Esters—The esters already reviewed have been the most studied because of their widespread clinical or commercial uses. Other nitrate esters have been investigated less thoroughly and usually only in comparison with the esters already discussed. In one such study with I and V, both esters were shown to form less than one nitrite ion per molecule in an enzymic reaction with reduced glutathione in rat liver mitochondrial suspensions (44). This observation substantiated previous results (18) that only 15% of the expected nitrite was produced from the reaction between V and tissue homogenates. The comparison of *in vitro* breakdown was investigated further by Needleman and Hunter (45) with incubations at 37°, employing 0.5 m*M* V, 3.1 m*M* IV, and 8.8 m*M* isosorbide dinitrate (XVII) with reduced glutathione and liver-soluble enzyme. TLC analysis of extracts shows that the three esters are degraded within 4 hr., with V and IV producing four nitrated metabolites and XVII producing one.

Similar results are found *in vivo* after intraperitoneal injection of 10 mg. of each nitrate in rats. Compound V is excreted unchanged with traces of three metabolites, while a small amount of intact IV is excreted with four clearly defined nitrated metabolites. Compound XVII is excreted primarily as one metabolite with traces of the parent compound and a second metabolite. None of the metabolites could be positively identified. Compound XVII was the subject of further studies *in vivo* in man and in the dog (46), where the orally administered ester did not appear in the 24-hr. urine. Only 1% of the dose



Scheme I—Diagram of the I transformation reaction coupled with the oxidized glutathione reductase assay system (reprinted from Reference 45)

appeared as the mononitrates, 2-isosorbide mononitrate (XVIII) or 5-isosorbide mononitrate (XIX), and it seems apparent that stepwise denitration led to a completely denitrated product.

In dogs, 1-chloro-2,3-propanediol dinitrate after oral administration is not detectable in the 24-hr. urine, but two metabolites are shown to be present by TLC on silica gel (47). These are the glucuronides, probably of the two mononitrates, formed by the partial denitration of the ester to give a free hydroxyl group available for conjugation with glucuronic acid.

MECHANISM AND SITES OF REACTION

Earlier studies showed that nitrate esters break down in tissues and that a reductive process is involved (12, 13, 18, 19), while the liberated inorganic nitrite is converted to nitrate by an oxidative reaction (15–17). One early attempt to postulate a mode of breakdown was by Krantz *et al.* (48), who suggested a reduction of the nitrate esters to the corresponding nitrite esters as the first step in metabolism, followed by hydrolysis to liberate inorganic nitrite. However, they discounted this theory after further studies, and it was left to Oberst and Snyder (18) to resurrect it as a result of their investigations with liver homogenates. The breakdown of the esters cannot be accounted for by hydrolysis to inorganic nitrate followed by reduction to nitrite, since it was shown that the latter reaction did not occur in the system investigated.

Needleman and Hunter (45), expanding upon the studies of Heppel and Hilmoe (19), showed that the reaction of nitrate esters with glutathione in the presence of the enzyme organic nitrate reductase is coupled with another enzymic reaction involving $NADPH_2$ to maintain a pool of glutathione in its reduced form. They presented the scheme shown in Scheme I; by showing that nitrite formation equaled $NADPH_2$ consumption, they were able to use the measurement of $NADPH_2$ disappearance to establish an assay system to determine the relative rates of enzymic activity with various nitrate esters as substrates. Under these conditions, V was shown to be the most actively transformed, followed by IV and then I. Compounds III, VI, and VII were shown to be transformed at comparatively very slow rates. The evidence suggests that reduced glutathione acts as a hydrogen donor for the reduction of nitrate esters, with two molecules of glutathione reacting with one of ester to liberate one molecule of inorganic nitrite. Williams (49) suggested that the overall reaction for a nitrate group may take place as follows:

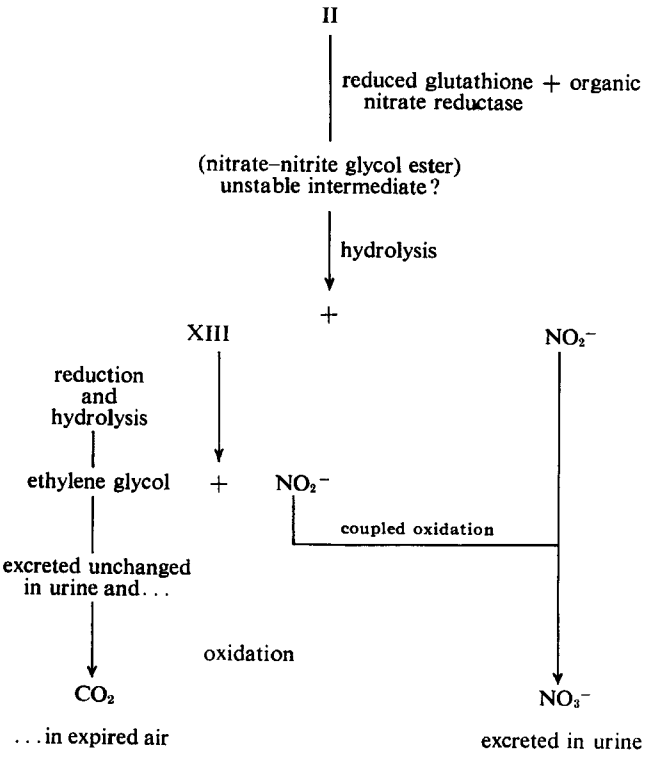
$$RO{\cdot}NO_2 \xrightarrow{+\,2H} RO{\cdot}NO \xrightarrow{H_2O} ROH + NO_2^-$$

The assumption, which was made by others (19), that an organic nitrite grouping exists before hydrolysis may be difficult to prove due to the short half-life involved, since it is known that compounds such as glycerol trinitrite decompose spontaneously in aqueous media (50). Efforts to detect a nitrite ester during studies on the metabolism of II were unsuccessful (40).

The enzyme responsible for the catalysis of the breakdown of nitrate esters was initially named "nitrite-forming enzyme" by Heppel and Hilmoe (19) and subsequently as "glycerol trinitrate metabolizing enzyme" by Needleman and Krantz (20) and then as organic nitrate reductase (45). This enzyme that catalyzes the glutathione–organic nitrate reaction was characterized (19, 20) and is listed by the Enzyme Commission under E.C. No. 1.8.6.1. as glutathione:polyolnitrate oxidoreductase (51). This enzyme is quite different from the nitroreductases, which act only under anaerobic conditions, and has been distinguished from the conjugating enzyme glutathione-*S*-alkyltransferase (20, 52). It is inconceivable, therefore, as was suggested (53), that glutathione may form conjugates with nitrate esters resulting in metabolites of the mercapturic acid type.

Organic nitrate reductase is widely distributed in mammalian tissues, but the location of the enzyme has been the subject of some debate. Studies with rat liver (44) and heart (37) showed the mitochondria to be most active in degrading organic nitrates, whereas in another study (20) the greatest activity was allocated to the 100,000×*g* soluble fraction of rat liver homogenate, with the mitochondria the least active. These differences may reflect differences in preparation of homogenate fractions and conditions of assay. Since the glutathione–organic nitrate reaction is linked to another enzymic reaction involving glutathione reductase (Scheme I), the activity of the latter enzyme presumably plays an important part in determining the activity of organic nitrate reductase. Glutathione reductase is present largely in liver and kidney and is most active in the 20,000×*g* supernatant fraction of rat liver homogenate but is almost wholly absent in mitochondria (54). The absence of a reduced glutathione-regenerating system in an assay for organic nitrate reductase could lead to the recording of low enzyme activity. The liver organic nitrate reductase system requirement for $NADPH_2$ and reduced glutathione does not always appear necessary for the reaction in other tissues. Degradation of III in heart subcellular fractions does not correlate with their glutathione content (37). Recently, it was shown that rat serum does not require $NADPH_2$ or reduced glutathione to degrade I (23). It is probable that two or more enzymes can be involved in reactions catalyzed by organic nitrate reductase. Fractions from column chromatography[2] of liver extracts from several species show two distinct peaks of enzyme activity (55), and their relative specific activities toward I, IV, and V as substrates are quite different. The possibility of two or more enzymes is also reflected by the difference between the rapid *in vivo* metabolism of XIII and its stability *in vitro* compared with the rapid breakdown of II in both systems (40).

[2] DEAE-Sephadex.



Scheme II—Metabolism of II in the rat

The oxidation of inorganic nitrite to nitrate in tissue homogenates is dependent upon the catalase content of the homogenate (17). Thus, kidney homogenate with about one-quarter of the catalase content of liver homogenate has one-quarter of the latter's oxidative capacity, and serial additions of catalase to a homogenate proportionately increase the oxidation rate of nitrite. It was demonstrated that the reaction is comparable to that shown by Keilin and Hartree (56) for the oxidation of alcohols and is catalyzed through a D-amino acid oxidase or xanthine oxidase system linked with catalase. Hydrogen peroxide formed by the oxidation is utilized by the catalase for the coupled oxidation of nitrite. The enzymic reduction of inorganic nitrate to nitrite in animal tissue is possible under anaerobic conditions (57), but aerobically this is extremely small compared with nitrite oxidation. The present picture of the reactions and possible mechanisms involved in the metabolism of a nitrate ester is depicted in Scheme II, which shows diagrammatically the breakdown of the II molecule in the rat.

ANALYTICAL CONSIDERATIONS

The determination of nitrate esters and partially denitrated breakdown products in biological materials may be classified under the headings colorimetric, TLC, and GLC analyses. In each case, the ester is extracted from the aqueous medium by an organic solvent and, after an intermediate step if necessary, submitted to the appropriate technique for its determination.

The most common procedure for colorimetric analysis of nitrate esters involves, after extraction, alkaline hydrolysis and colorimetric measurement of liberated

nitrite. This basic procedure has been used by investigators for at least the last 40 years (58–61). Ethyl ether is the solvent generally employed for extraction, and the efficiency of removal is 100% in some cases. The next steps, hydrolysis and colorimetric determination, may be most conveniently carried out on the ether extract directly (59, 61). Hydrolysis does not liberate a constant percentage of nitrite from all esters, but each ester liberates a constant proportion under standard conditions of time, temperature, and alkalinity. By varying the alkalinity, it is possible to carry out a differential hydrolysis to distinguish between two esters (60). The nitrite liberated by the hydrolysis step is measured colorimetrically by a diazotization and coupling reaction, sulfanilic acid or sulfanilimide being used as the initial agent and 1-naphthylamine or *N*-(1-naphthyl)-ethylenediamine as the coupling agent. The overall procedure provides a relatively sensitive method for those esters readily hydrolyzed, II and I being detectable at the 0.5-mcg./ml. level.

The colorimetric methods are virtually nonspecific, apart from a few cases where differential hydrolysis can be applied. This is not a disadvantage if only one ester is present, but cases arise during metabolism when mixtures of esters are present due to partial denitration of the parent nitrate ester. Chromatographic methods overcome the latter difficulty, provide adequate separation, and, at the same time, maintain sensitivity of detection. Di Carlo *et al.* (26) developed a TLC separation for III and its four metabolites, which they used as a basic procedure throughout their studies on the metabolism of III. The products of metabolism are removed from urine or tissue by extraction with ether, methanol, or dioxane. After concentration, aliquots are spotted onto 0.25-mm. silica gel G plates, and the chromatograms are developed using a toluene–ethyl acetate–butanol–water (10:5:2:2) solvent system (31). The nitrates are detected on the plates by spraying with 1% diphenylamine and subjecting to UV radiation for a few minutes with the appearance of blue spots (62). By utilizing ^{14}C-labeled III, the ester and its four metabolites can be identified and measured after TLC by a combination of radioscanning and nitrate assay. Similar methods were applied to the determination of I and the di- and mononitrate metabolites (63). TLC followed by identification with diphenylamine spray reagent has been used for urinary analysis in metabolism studies on I (20), XVII (46), and 1-chloro-2,3-propanediol dinitrate (47).

In 1960, Evered and Pollard (64) showed that nitrate esters could be separated on a GLC column, and this technique has been applied to the determination of II, XIII, XIV, XVI, XV, XVII, and I in urine and tissues (61, 65–67). Extraction procedures are simple, involving shaking the sample for a few minutes with an organic solvent and injecting microliter quantities of the extract onto a column with a 5 or 10% silicone stationary phase at a temperature between 100 and 140°. The electron-capture detector is normally used for measuring the separated components, since it is much more sensitive and selective for nitrate esters than the flame-ionization detector. It is possible to measure 0.01 p.p.m. nitrate ester in a sample by this technique, which means that the metabolism of minute doses of ester can be followed.

Inorganic nitrite and nitrate arising from the metabolism of nitrate esters can be determined by a variety of methods. Crandall (13) used the Griess–Ilosvay colorimetric procedure to measure nitrite produced by I breakdown in perfusates. Stieglitz and Palmer (68) made a thorough study of the application of this method for the determination of trace amounts of nitrite in blood. The deproteinized sample is analyzed using 1-naphthylamine and amino-G acid for color development. To attain the sensitivity needed for the measurement of the very low normal blood nitrite concentrations (about 1 mcg./100 ml.), a sample volume of 8 ml. is required. Apart from the introduction of *N*-(1-naphthyl)ethylenediamine as a faster coupling agent (69), the basic method described here has been used with only minor modifications to the present day by investigators studying the appearance of nitrite during metabolic reactions of nitrate esters (18, 19, 25, 44, 70, 71).

Although the determination of inorganic nitrite in blood, urine, and tissue homogenates has proved to be straightforward, the measurement of inorganic nitrate has not. This is due basically to the inherent difficulties of the colorimetric determination of small amounts of nitrate ion, the methods being prone to interference and lacking specificity. The methods can be divided into direct and indirect procedures, the latter depending upon the reduction of nitrate to nitrite prior to colorimetric analysis. The direct methods involve *m*-xylenol (17), phenoldisulfonic acid (72), or brucine (59) as reagents. Among reducing agents for the indirect method are zinc dust in the presence of ammonium hydroxide and a manganous chloride catalyst (73, 74) and the novel use of bacterial nitratase (75).

Automated methods are especially useful for the rapid analysis of large numbers of samples arising from metabolism studies or clinical trials. A method was described using the Technicon AutoAnalyser (76), whereby the dialysis unit separates nitrite and nitrate from blood protein and eliminates any preliminary manual deproteinization. Nitrite in a sample is first measured by a diazotization and coupling reaction, while nitrate is reduced to nitrite by a zinc column incorporated into the system prior to color formation. The recovery of added anion to blood is reproducible and almost 100%.

There should be no delay in the determination of nitrate esters and metabolites on samples submitted for analysis, since further breakdown or other changes can take place rapidly *in vitro* (61, 76).

METABOLISM AND PHARMACOLOGICAL EFFECTS

Hay (77), in 1883, recognized that the therapeutic action of I bore a strong resemblance to that of inorganic nitrites, and he suggested that the mode of action of this ester arose from its breakdown to nitrite. This assumption was soon discounted (78) on the grounds that the amount of nitrite so produced was not enough to account for the potent vasodilating power of I; another theory was put forward that the physiological action of this ester is due to the intact molecule (79). The latter observations were endorsed by later work in this field, where the administration of minute doses of 60 mcg./kg.

IV or I injected intravenously in dogs produced a marked decrease in blood pressure with no increase of blood nitrite above control values (70). Similarly, after the oral administration of III to man, the levels of blood inorganic nitrite do not correlate with physiological responses, although the interesting suggestion arising from this study is that blood nitrite levels may not reflect the intracellular concentrations where the pharmacological effects are initiated (25). It is possible that the nitrate ester acts as a "carrier" and releases nitrite intracellularly. In studies with II in the rat (40), the time course of metabolism manifest by XIII and inorganic nitrite and nitrate production was compared with recordings of blood pressure, which is depressed very rapidly and almost maximally after II injection and before any significant breakdown of the molecule can have occurred. The delayed return of the blood pressure to normal can be attributed only in some small part to the metabolites XIII and inorganic nitrite. The blood pressure response to XIV and its metabolites is very similar (41).

Attempts to link the action of nitrate esters at the cellular level with observed pharmacological effects still occupies the attention of several investigators. It was established (80) that there is a relationship between the ability of nitrate esters to stimulate mitochondrial respiration, their rate of enzyme reaction with glutathione, and their oil–water solubility. The more water-soluble nitrates, such as VIII, show little or no reaction with reduced glutathione and have no effect on mitochondrial respiration in contrast to the more lipid-soluble esters which have much greater activity in both respects. It was also shown that those esters more rapidly denitrated in the glutathione-dependent reductase reaction are the more potent vasodilators (81) and that lipid solubility is not such a good index of vasodepressor activity when the molecular configuration is such that the reaction is blocked, as with long-chain derivatives of I.

It is unlikely that the ATP–ATP-ase reaction is the target enzyme system of nitrate esters, since the correlation of vasodilator action with ATP-ase inhibition obtained on rabbit aorta could not be repeated in the uterus or skeletal muscle (82) nor in other studies (83) with coronary ATP-ase in dogs. Sodium nitrite and some nitrate esters are uncouplers of oxidative phosphorylation in rat liver mitochondria (84), with I and IV being much less effective than V. These differences are not due to differences in rates of metabolism, since the enzyme-catalyzed reaction with glutathione in mitochondrial suspensions is greater with I than with the other two nitrates (44). Since I is the more potent of these three vasodilators, any relationship between vasodilation and uncoupling of aerobic phosphorylation remains questionable.

Compounds I, II, and XIV are monoamine oxidase inhibitors (85–87), but their metabolites have no such effect.

Contradictory evidence was obtained from the limited number of studies carried out concerning the relationship between metabolism and the onset of tolerance to the repeated administration of nitrate esters. Crandall (13), using *in vitro* assays, showed that blood from dogs made tolerant to II degrades nitrate esters more slowly than controls, while Hasegawa and Sato (39), with a small number of experiments in rabbits, indicated that the metabolism of II increased in rate after repeated administration of the ester. In more detailed studies with II (88) in the rat, it was shown that the metabolism of this ester, manifest by blood levels and urinary excretion of metabolites, is unaltered after daily administration for 2–8 weeks. It was suggested that the tolerance that develops to the cardiovascular effects of these esters may be due to the changing influence of a physiological compensatory mechanism rather than to any change in the pattern of metabolism.

The production of methemoglobin due to the action of nitrate esters on blood has received limited attention. The lack of interest is shown by the fact that Bodansky (89), in a comprehensive review of compounds forming methemoglobin, did not mention nitrate esters. This effect was noted toward the end of the last century (12, 90), but it was not until the mid-1930's, when spectrophotometric methods sensitive to less than 1% methemoglobin were developed (91, 92), that the way was opened for more precise studies. Experiments with cats (93) showed II to be 10–20 times more effective than XIII and 4 times as effective as I in producing methemoglobin. By taking into account the ratio of ester given to the amount of methemoglobin formed, it was concluded that the reaction is molecular and not catalytic, as is the case with aromatic nitro compounds. The injection of an LD_{50} of XIV in the rat brings about almost complete conversion of hemoglobin to methemoglobin, and the cause of death can be due simply to anoxia brought about by the destruction of the oxygen-carrying power of the blood (41).

CONCLUDING DISCUSSION

Early studies indicated that nitrate esters break down rapidly in contact with animal tissue, with the production of inorganic nitrite. The introduction in recent years of more sophisticated methods of analysis, such as TLC and GLC, and the use of radiolabeling have facilitated the detection and determination of metabolites in addition to inorganic nitrite and nitrate. Together with automated procedures for the latter, these methods have ensured the attainment of a more comprehensive picture of the progress of metabolism by enabling several parameters to be measured collectively and quickly.

Although the production of inorganic nitrite has been universally demonstrated, Hasegawa and Sato (39, 94) claimed that none is detected after the injection of large doses of II in the rabbit. Their main evidence for the absence of nitrite devolves upon observations of the type of methemoglobin produced in the blood *in vivo* compared with that *in vitro* where II and nitrite are shown to form methemoglobins with different absorption maxima at 500 and 540 nm., respectively. It is doubtful if this criterion can be applied *in vivo*, bearing in mind the number of parameters affecting methemoglobin formation and reduction (95).

Detailed *in vivo* metabolic studies of I, III, II, and XIV show that the parent molecules are denitrated rapidly and do not appear in significant quantities in rat urine after 24 hr. Originally, it was thought that I was

resistant to further degradation after the removal of one nitrate group (20). However, recent evidence (21) shows I to have the same pattern of breakdown as the other esters, which is denitration stepwise to the parent alcohol. This process is slowest with III where the pentaerythritol so formed does not break down to carbon dioxide, as do the other alcohols, but is excreted unchanged.

In vitro, I, II, and XIV lose only one nitrate group, the reaction stopping at this point since VI and VII or mononitrates so formed are relatively stable under the experimental conditions. In contrast, III is degraded as far as XI (37), although this apparent difference may be due to the length of time the reactions were studied. In whole blood *in vitro*, III loses only one nitrate group after 2 hr. of incubation, which compares with II breakdown (40); it is probable that under strictly comparable conditions, the breakdown of the four nitrate esters *in vitro* is very similar. There is contradictory evidence concerning the fraction of blood most active in degrading nitrate esters. Several studies showed that all the activity resides in the red cells, with little or no reaction in plasma (13, 40, 41). Other investigations, however, indicate appreciable breakdown of I in rat serum and plasma (23, 24), and it was shown that III is significantly denitrated in human plasma, although to a lesser extent than in red cells (27).

Although radiochemical studies with nitrate esters can account for the fate of ^{14}C during metabolism, only 60% could be recovered in studies to account quantitatively for the fate of the nitrate groupings (40, 41). It is possible that —OH groups formed after denitration can conjugate with glucuronic acid and remove partially denitrated products by this route, although it seems that this is only a minor pathway (47). It is conceivable that ester nitrate nitrogen may be eliminated by pathways such as reduction to ammonia or elemental nitrogen, and exploratory studies using the heavy isotope ^{15}N can be envisaged.

It has been established that the metabolism of nitrate esters proceeds *via* an enzyme-catalyzed reaction with reduced glutathione, the latter acting as a hydrogen donor for the reductive process. The enzyme, organic nitrate reductase, is present in many tissues, although its subcellular location was the subject of some controversy (20, 37, 44). Needleman and Hunter (45), by establishing an assay system containing liver organic nitrate reductase and glutathione coupled with another involving $NADPH_2$ and glutathione reductase to maintain glutathione in a reduced form, were able to relate the low enzymic transformation of VI and VII with their relative stability *in vivo*. Such relationships do not necessarily hold for other nitrate esters since III and XIII, which have little or no activity *in vitro*, are both actively transformed *in vivo* (29, 40). These differences suggest that more than one reaction may be involved in their metabolism. Recently, it was suggested that rat serum contains an enzyme which will catalyze nitrate ester reduction in the absence of glutathione (23), and other evidence shows the presence of two organic nitrate reductases in liver (55).

According to current theory, the enzyme-catalyzed reduction of nitrate esters proceeds *via* the formation of a nitrate–nitrite ester, the nitrite moiety hydrolyzing to form an —OH group and releasing inorganic nitrite. The reaction proceeds on consecutive nitrate groupings until full denitration has occurred. Although this has not been substantiated experimentally, no other feasible suggestion has been put forward.

The quest for the link between metabolism and pharmacological action of nitrate esters is still the subject of many studies. All the evidence suggests that the direct action of the intact molecule is responsible for effects such as vasodilatation, and the only effect of metabolism seems to be to terminate such action. The onset of tolerance to these esters does not seem to arise from an altered metabolism (88). It is apparent that the oil–water partition coefficient (2, 80, 96, 97) plays a part in differentiating the pharmacological potency of different nitrate esters, and it is evident that the more water-soluble metabolites are not as effective as the parent ester. The more prolonged therapeutic action of III compared with I is attributed in part to its binding to nonenzymic protein molecules and demonstrates another factor contributing to differences in pharmacological action (98).

Despite the impressive evidence for the direct action of the intact molecule, it is still possible that metabolism occurring intracellularly may present a different picture from that shown by circulating blood levels. Inorganic nitrite liberated in a cell from a lipid-soluble nitrate ester may have a much more potent effect than when it is administered alone and is unable to enter cells to the same extent. Similarly, the question remains whether methemoglobin is produced by the intact molecule or by inorganic nitrite released by denitration.

The study of the metabolism of nitrate esters has slowly evolved during the last 80–90 years in a rather uncoordinated fashion. Recent investigations added greatly to the sum of knowledge and clarified many points, but they also raised new problems of interpretation of the facts. Apparent differences between the metabolism of individual esters can be resolved only by a comprehensive study of their breakdown under comparable routes of administration, dose levels, times of study, and analytical procedures and by taking into account such parameters as oil–water partition coefficients and the effects of protein binding.

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