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Pentaerythritol tetranitrate metabolism: a non-essential role for the flora*

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Pentaerythritol tetranitrate (PETN) is one of a number of nitrate esters used for the treatment of angina pectoris. After oral administration of PETN, the sequential removal of nitrate groups leads to the formation of similar metabolic products in both man and rat. Pentaerythritol (PE) and its mononitrate are the predominant metabolites found in the urine, while the feces contains mainly PE and unmetabolized PETN [1–3]. The plasma contains these and a variety of other metabolites but relatively little PETN [3, 4]. The obligate metabolic reaction in the formation of these products is the loss of one nitrate group. The available metabolic and pharmacokinetic evidence suggests that this reaction occurs in the gastrointestinal tract [1, 5] and that the conversion of the water-insoluble PETN to the water-soluble PE-trinitrate at this site governs the subsequent metabolism and disposition of PETN.

There are at least two interesting implications of the suggestion that the denitration of PETN can be ascribed to the metabolic activity of the gastrointestinal flora [1, 5]. One is that the release of the absorbable PETN metabolites would then be controlled by the bacterial flora or enzymes released by the flora. The second is that the intestinal bacteria would then release nitrite from this drug which could react with secondary amines derived from dietary constituents [6] or perhaps from drugs containing substituted amines to form nitrosamines. Carcinogenic nitrosamines can be formed *in vitro* when certain strains of bacteria are incubated with nitrite and various substituted amines [7, 8]. However, the physiological importance of this reaction remains uncertain since bacteria and both nitrite and secondary or tertiary amines are not ordinarily found at a single site in the body.

This investigation, undertaken to elucidate the role of the intestinal bacteria in the release of nitrite from PETN, has disclosed no evidence that the intestinal bacteria participate in this reaction. In addition there appears to be little difference in the absorption and metabolism of PETN between germ-free and conventional animals.

Materials. PETN, a gift of the Warner Lambert Research Institute, Morris Plains, NJ, (Lot No. 11251) was supplied as a mixture with 5 parts by weight of lactose. Radioactive PETN (1 part of [^{14}C]PETN with 7 parts of lactose) was generously supplied by Dr. F. J. Di Carlo and M. E. J. Merrill of the Warner Lambert Research Institute. The specific activity of this mixture was 0.62 μCi/mg.

Animals. Conventional and germ-free male rats of the Sprague–Dawley strain weighing 170–200 g were used (Charles River Breeding Laboratories, Wilmington, MA). All animals were housed in metabolism cages (Acme Research Products, Cleveland, O) which permitted separation of urine and feces. Germ-free rats were maintained in metabolism cages within a sterile isolator (Standard Safety Equipment, Palatine, IL) as previously described [9]. All animals received the same diet (4RF; Country Foods, Syracuse, NY); for the germ-free animals, food was sterilized by irradiation through the kindness of the Natick Army Labs, MA.

Animal experiments. PETN was removed from lactose by extraction with acetone. The extract (diluted to contain 10 mg PETN/kg of body wt for each rat) was mixed with the powdered diet; the acetone was allowed to evaporate before the mixture was made accessible to the animals. In all cases, the supplemented food was consumed by the animals within a 15-hr period. When radioactive PETN was fed, the specific activity was 1·2 μCi per mg of PETN. On this regimen each animal received approximately 4×10^6 cpm.

Feces and urine were collected continuously, and removed from the isolators at 18, 42 and 67 hr (unless otherwise noted) for immediate analysis as described below. Animals were sacrificed by decapitation before dissection of their gastrointestinal tracts. The entire contents of the small intestine, cecum and colon were analyzed immediately.

Bacteriology. Cecal contents (weighing approx 1 gm) were removed aseptically from rats which had been killed by decapitation and were added to 10 ml of prereduced sterilized N_2C broth [9] and incubated anaerobically for 16 hr at 37°. At this time 0.5 ml of this culture was added to 1.0 mg of [^{14}C]PETN (sp. act. 2.8 μCi/mg PETN; dissolved in 0.3 ml of ethanol) in 10 ml of N_2C broth. This reaction mixture, and a control lacking the bacterial inoculum, were incubated in an oxygen-free atmosphere at 37°. To determine the metabolism of PETN, samples (2.0 ml) were removed for analysis at 24 and 72 hr. [^{14}C]PETN and its metabolites were extracted from these samples by shaking with four 2.0 ml portions of ethyl acetate. The extracts were combined and, after concentration under a stream of nitrogen, were dissolved in 0.03 ml of ethyl acetate for application to t.l.c. plates. Only 1 per cent of the radioactivity in the preparation of [^{14}C]PETN remained in the aqueous phase of suspensions after this extraction procedure.

Measurement of nitrite. Nitrate was assayed as nitrite by a modification of the method of Schneider and Yeary [10]. Urine samples were extracted with ethyl acetate to remove the organic nitrate esters of PE. Then 1.0 ml of extracted urine was mixed with 0.2 ml of 10% $HgCl_2$ and allowed to stand for 3 min. After the addition of 0.25 ml of 1.0 M Na_2CO_3, the tubes were mixed, allowed to stand for 2 min and centrifuged at approximately 2500 *g* for 5 min. One ml of the supernatant solution was diluted with an equal volume of distilled water and treated with 0.5 ml of an NH_4Cl solution (50 g of NH_4Cl dissolved in 500 ml of distilled water, and adjusted to pH 9.6 with NH_4OH before dilution to 1 liter with water) and 1 g of a wet suspension of particulate metallic cadmium (prepared as described by Schneider and Yeary [10]). The mixtures were shaken by hand for 10 min, centrifuged at 2500 *g* for 5 min, and samples of the supernatant solution diluted to 3.0 ml with water. A solution of sulfanilamide (0.5% sulfanilamide in 50% HCl; 0.3 ml) was added, followed by 0.1 ml of 5% N-(1-naphthyl)ethylenediamine dihydrochlor-

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ide in water. After mixing, the solution was allowed to stand for 20 min and then its spectral absorbance at 540 nm was determined on a Perkin–Elmer spectrometer (model 44). The absorbance readings were related to a standard curve obtained following the above protocol with known additions of $NaNO_2$ to urine since the standard curve in the presence of urine was slightly lower than that obtained in the absence of urine. This method yielded a linear calibration curve with an absorbance of 0.6 for 18 μg $NaNO_2$, and a urine blank was negligible for conventional rats.

Extraction of [^{14}C]PETN products. PETN and its tri-, di- and mono-nitrated derivatives were extracted from the total samples of urine and feces with 25 ml of ethyl acetate. For this procedure the feces-containing mixture was homogenized for 30 sec and the urine samples were shaken manually for 2–3 min. After centrifugation at 2500 g for 5 min, the ethyl acetate was collected and the procedure repeated three additional times using 15-ml aliquots of ethyl acetate. The ethyl acetate extracts from each sample were combined, and the radioactivity quantified on a 0.2-ml aliquot. Radioactivity was assayed in a Packard Tri-Carb liquid scintillation spectrometer (model 3003) using 15 ml of a scintillation mixture with the following composition per liter: 2,5-diphenyloxazole, 4.0 gm; 1,4-(bis[2-(5-phenyloxazolyl)]-benzene, 50 mg; toluene, 980 ml and methanol, 20 ml.

Glucuronide derivatives of PETN metabolites are not soluble in ethyl acetate [4] and were extracted from the fecal residue with water as described above. To determine the radioactivity in the glucuronide fraction an aliquot (0.1 ml) of the aqueous extract of the fecal residue or of the urine sample after ethyl acetate extraction was assayed for radioactivity in 15 ml of Aquasol Universal Cocktail (New England Nuclear Corp.). Extraction by this procedure was essentially complete since the extracted fecal residue, which had been dried in air and then heated at 60° for 5 min with 0.2 ml Soluene® (Packard Instrument Corp., Downer's Grove, IL) contained only 0.1 per cent of the fed radioactivity when assayed in 15 ml of Aquasol solution.

Greater than 95 per cent recovery of [^{14}C]PETN was demonstrated by these extraction procedures when known quantities were added to urine or feces.

Thin layer chromatography. Aliquots of the ethyl acetate extracts of urine and feces (20–50 ml) were concentrated under a stream of nitrogen at 40°. The dark-green residues were redissolved in ethyl acetate (0.5 ml) and 1–2 μl samples were subjected to ascending t.l.c. on pre-coated Silica gel GF plates (Analtech, Inc., Newark, DE). The solvent system consisting of toluene–ethyl acetate–butan-1-ol–water (10:4:2:2 by vol; upper phase) was modified from that described by Di Carlo *et al.* [2] to afford a greater separation of the tri- and dinitrate metabolites. After development, the plates were air-dried, and the areas of sample separation divided into approximately fifteen sections each of which was removed from the plate as a powder and assayed for radioactivity in 15 ml of toluene–methanol scintillation fluid. The relative mobility of compounds on t.l.c. plates was compared to products obtained after [^{14}C]-PETN was heated in 1 N HCl at 100° for 4 hr. The degradation products of PETN in turn were identified on the basis of their relative mobility as described by Di Carlo *et al.* [2] for solvent system J. In this procedure nitrate esters were revealed as dark-blue spots by spraying with 1% diphenylamine in ethanol, and subjecting the plate to u.v. light for 1 min. The following R_f values were obtained: PETN 0.93; PE-trinitrate 0.74; PE-dinitrate 0.66; PE-mononitrate 0.29.

To determine whether the intestinal microflora is required for the release of nitrate from PETN, germ-free and conventional rats were fed PETN and the pattern of nitrate excretion in the urine was examined (Fig. 1). Since nitrate excretion in the urine of these germ-free rats does not differ significantly from that in the conventional rats, the possibility is excluded that the flora has an obligate role in the release of nitrate from PETN in the conventional rat.

After incubation of cecal contents with [^{14}C]PETN as described in Materials and Methods, the degradation of the drug after 72 hr was only 4 per cent compared to an apparent degradation of 5 per cent in the control incubation mixture which lacked bacteria.

In a sense, these experimental results are the opposite of those previously used to establish an obligate role for the flora in the metabolism of certain other exogenous

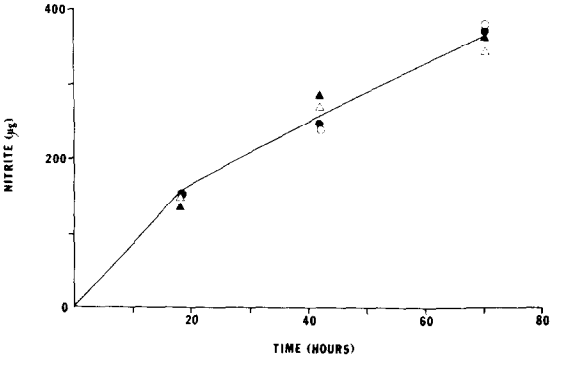

Fig. 1. Recovery of nitrite in the urines of conventional and germ-free rats after oral administration of PETN. The accumulated nitrite recovered from conventional rats 1 (○) and 2 (●) and germ-free rats 1 (▲) and 2 (△) are shown for the times indicated.

Table 1. Recovery of ^{14}C label after oral administration of [^{14}C]PETN to germ-free and conventional rats

Specimen	Animal designation		Radioactivity Recovered (%) 18 hr	42 hr	67 hr
Urine	Germ-free	1	15.4 (11.1)*	16.9 (12.1)	17.9 (12.8)
		2	16.2 (11.5)	18.6 (13.1)	19.4 (13.7)
	Conventional	1	11.5 (7.0)	13.3 (8.1)	13.6 (8.1)
		2	5.8 (3.8)	8.7 (5.7)	9.0 (5.7)
Feces	Germ-free	1	9.9 (8.5)	18.5 (15.8)	21.2 (18.3)
		2	8.1 (6.5)	16.6 (13.5)	19.8 (16.2)
	Conventional	1	11.2 (4.2)	24.9 (6.5)	25.1 (8.9)
		2	13.5 (5.4)	27.5 (9.0)	29.4 (10.7)

* Data from each of two germ-free and two conventional rats are expressed as the total cumulative per cent of radioactivity recovered at the time indicated, followed by the cumulative per cent of water-soluble products in parenthesis.

Table 2. Recovery of [^{14}C]PETN and its metabolites from excreta of germ-free and conventional rats

Specimen	Animal designation		Time	PETN	PE-trinitate	PE-dinitrate	PE-mononitrate
Urine	Germ-free	1	18 hrs.	2.0*	0.5	19.0	74.0
		2	42 hrs.	8.0	4.0	11.0	73.0
	Conventional	1	18 hrs.	12.0	1.0	11.0	73.0
		2	42 hrs.	8.0	3.0	6.0	82.0
Feces	Germ-free	1	18 hrs.	8.0	6.0	25.0	41.0
		2	42 hrs.	6.0	7.0	21.0	57.0
	Conventional	1	18 hrs.	40.0	21.0	28.0	0
		2	42 hrs.	40.0	27.0	22.0	0

* Data on each of two germ-free and two conventional rats are expressed as the per cent of the total radioactivity in the ethyl acetate extracts of samples obtained at the times indicated.

compounds. The dehydroxylation at the 4-position of caffeic acid [11] and of dopamine[12] occurs in the conventional rat but not in the germ-free rat. Similarly, the reduction of the azo bond in salicylazosulfapyridine [13] and of the nitro group in *p*-nitrobenzoic acid [9] occurs in the conventional rat but not in the germ-free rat. In each of these instances the metabolic reaction lacking in the germ-free rat was demonstrated either in pure or mixed cultures of the flora. Thus, failure to demonstrate a difference in PETN metabolism between germ-free and conventional rats and failure to demonstrate PETN metabolism in cultures of the gastrointestinal flora may be taken as evidence to exclude a role for the flora in the initial release of nitrate from PETN.

The comparison between germ-free and conventional rats and the *in vitro* bacterial experiments on PETN do not exclude the possibility that the flora may release nitrate from some of the metabolites of PETN such as the tri-, di- and mono-ester. To determine whether the flora might effect the metabolism of PETN beyond the initial release of nitrate, the metabolites were examined after radioactive PETN was fed to germ-free and conventional rats. For both germ-free and conventional rats, the total recovery of PETN and its metabolites in urine and feces (Table 1) is approximately 40 per cent. The urine of the germ-free rats at 18 hr and the feces for up to 38 hr contain a relatively greater amount of water-soluble metabolites than are found in the conventional rat. This can be attributed to the absence of bacterial β-glucuronidase in the germ-free rat [14] and the consequent retention of a higher fraction of metabolites as their glucuronide derivatives.

With regard to the distribution of the nitrate esters, the only significant difference between the two types of rat is that conventional rats tend to have more PETN and the triester in the feces whereas the monoester seems to predominate in the feces of the germ-free rat (Table 2). This distribution suggests that the flora may play a role only in the removal of the nitrate group from PE-mononitrate.

Because PETN is insoluble in both water and non-polar solvents, the intestinal flora [1, 5] or bacterial enzymes in the gastrointestinal tract [15] have been invoked to explain its mode of absorption from the intestine. The results presented here seem to exclude this possibility and to suggest therefore the importance of nitrate release by mammalian systems previously demonstrated in plasma, erythrocytes, and in subcellular fraction of the heart and liver [16–18]. The metabolism of PETN by the gastrointestinal mucosa may provide a source of nitrite which, together with substituted amines and colonic bacteria could lead to the formation of nitrosamines in the colon.

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