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BILIARY EXCRETION AND BIOTRANSFORMATION OF PENTAERYTHRITOL TRINITRATE IN RATS

MALCOLM C. CREW, ROSEMARIE L. GALA, LLOYD J. HAYNES and FREDERICK J. DI CARLO

Dept. of Drug Metabolism, Warner-Lambert Research Institute, Morris Plains, N.J., U.S.A.

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Abstract—A study was made of the biliary excretion of ^{14}C-pentaerythritol trinitrate in the rat to elucidate the possible role of enterohepatic circulation in the prolonged action of the drug. The biliary drug excretion consisted entirely of metabolites which were previously unknown, namely, the glucuronides of pentaerythritol mononitrate, dinitrate and trinitrate. Drug radioactivity entered the bile faster after intravenous than after oral dosing. This difference (51% vs. 30% of the dose in 4 hr) was entirely due to pentaerythritol dinitrate glucuronide; the pentaerythritol trinitrate glucuronide level was approximately the same (9·5% of the dose in 4 hr). Enterohepatic circulation was indicated by the observation that 2·5 times more drug radioactivity was excreted into the urine by intact rats than by biliary cannulated rats. A comparison of the biliary and urinary excretion products of pentaerythritol trinitrate suggests that the enterohepatic circulation provides the opportunity for gastrointestinal resorption and recirculation of the free drug.

THE PROLONGED hypotensive activity of pentaerythritol (PE) trinitrate[1] suggested its possible enterohepatic circulation. This problem was investigated in the rat with the ^{14}C-labeled drug so that biotransformational alterations could be followed by thin-layer chromatography (TLC) and radioscanning techniques developed earlier.[2] The urinary as well as the biliary drug excretion was studied qualitatively and quantitatively after the oral and intravenous administration of ^{14}C-PE trinitrate to biliary cannulated rats. Blood assays were performed in order to ascertain whether drug transport into the bile was an active or passive process, and the intravenous dose was varied to determine whether or not the transport process was saturable. Unoperated rats were dosed by both routes for comparison of the urine data, and undosed bile fistula rats were studied to measure normal bile flow rates. Additionally, the biliary excretion of unsubstituted PE, the end product of PE trinitrate metabolism, was examined to assess its possible contribution to the enterohepatic circulation of the trinitrate metabolites.

MATERIALS AND METHODS

^{14}C-Pentaerythritol (PE) trinitrate. This compound was synthesized from pentaerythritol-1,2-^{14}C at the Hercules Research Center. Assay by TLC indicated the product to contain 97·3% PE trinitrate, 2·1% PE tetranitrate and 0·6% PE. The synthetic preparation was dispersed in 19 parts by weight of c.p. lactose. The sp. act. of the pentaerythritol trinitrate–lactose mixture was 0·302 mc/g.

^{14}C-Pentaerythritol. This compound was synthesized with a sp. act. of 1·9 mc/g.

Animals. Male CFN Wistar rats (Carworth Farms) weighing 330–370 g were employed. After treatment the animals were housed individually under restraint in

glass metabolism cages (Aerospace Industries, Inc., Garnerville, N.Y.) without food or water.

Bile fistula rats. Cannulas of PE 50 polyethylene tubing were inserted into the bile ducts of the rats using the procedure described by Fisher and Vars.[3] A 27-in, length of tubing was used in each case. The rats were placed in the metabolism cages to observe the initial bile flow. The doses were administered 1 hr following the operation only to those animals which did not exhibit unusually low bile flows. After the administration of the dose the bile collection vials were replaced at 5-min intervals for the first 2 hr and then at 0·5-hr intervals up to 5 hr. After 20 hr the vials were replaced every hour for 7 hr. In addition to the collections in the vials, very small aliquots (5–20 μl) of bile were collected from the end of the tube after the initial 2-hr period. These small aliquots were taken at 10–20 min intervals in the earlier time periods and at hourly intervals after 20 hr. In the case of one animal in each group, additional collections were made in a similar manner in the 7–14 hr interval. By this method of sampling it was possible to determine the rate of bile flow, the rate of biliary ^{14}C excretion and the cumulative ^{14}C excretion at desired intervals. At the conclusion of each experiment the cannula was removed and its internal volume measured (130–160 μl) to allow for the correction of sample dwell time in the tube.

Experimental groups. The following experimental groups of rats were studied: (Group A) Bile was collected at intervals over a period of 0–4 hr from 3 bile fistula rats which received no drug. (Group B) ^{14}C-PE trinitrate (10 mg/kg) was administered intravenously (tail vein) to 3 bile fistula rats. Collected for assay were a series of bile samples and the 0–24 hr urine from each rat. (Group C) ^{14}C-PE trinitrate (10 mg/kg) was administered intravenously to 3 unoperated rats and their urine was collected from 0 to 24 hr. (Group D) ^{14}C-PE trinitrate (10 mg/kg) was administered intravenously to 3 intact rats. Blood samples were obtained by heart puncture for total ^{14}C assay at 15 min intervals from 5 to 65 min after dosing. (Group E) ^{14}C-PE trinitrate (10 mg/kg) was administered intravenously to 3 rats with ligated bile ducts. Blood samples were obtained as for Group D. (Group F) ^{14}C-PE trinitrate (0·05 mg/kg which is in the clinical dose range) was administered intravenously to 3 bile fistula rats and a series of bile samples was collected for only 4 hr. (Group G) ^{14}C-PE trinitrate (10 mg/kg) was administered by gavage to 5 bile fistula rats. Bile and urine specimens were collected for ^{14}C counting. Bile and urine from rats 1, 2 and 3 were also assayed for metabolite composition. The gastrointestinal tracts were excised at 24 hr from rats 3, 4 and 5. These tissues were homogenized and counted to determine residual ^{14}C. (Group H) ^{14}C-PE trinitrate (10 mg/kg) was administered by gavage to 3 intact rats. The 0–24 hr urines were collected for assay. (Group I) ^{14}C-PE (10 mg/kg) was administered intravenously to 2 bile fistula rats. Biles and urines were collected for assay. (Group J) ^{14}C-PE (10 mg/kg) was administered intravenously to 9 intact rats and blood samples were obtained as for Group D.

Radioactivity counting. Quantitative assays for ^{14}C were conducted with the use of a Packard Tri-Carb Model 3020 Liquid Scintillation Spectrometer. Samples were mixed with 18 ml of scintillation solution which consisted of 7·0 g PPO (2,5-diphenyloxazole), 0·3 g dimethyl POPOP [1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene], and 100 g naphthalene in 1·0 litre dioxane.

Bile samples were prepared for counting by dissolving 5–20 μl of bile in 0·5 ml

of methanol. Urine samples were diluted 1 : 10 and blood samples were diluted 1 : 100 with water. Aliquots (1·0 ml) of the diluted solutions were counted. The gastrointestinal tracts excised from the rats in Group G were homogenized with 150 ml of 75% dioxane. The residue after filtration was extracted twice with 150-ml portions of 75% dioxane. The filtrates were combined and aliquots removed for counting.

Thin-layer chromatography. All of the urine samples and selected bile samples were submitted to TLC to separate the radioactive components. The chromatograms were run on 2 × 8 in. plates coated with 250 μ of Silica G. After development in a solvent, the radioactivity on the plates was located and evaluated using a Packard Model 7201 Radiochromatogram Scanner. Quantitation was obtained from the peak areas on the scanner tracings. The solvent systems used for development of the bile chromatograms were solvent 1, *n*-butanol–conc. NH_4OH–water (4 : 1 : 3, upper layer) and solvent 2, *n*-butanol–acetic acid–ether–water (6 : 3 : 9 : 1). In addition to these two solvents the urines were examined using solvent 3, toluene–ethyl acetate (1 : 1) and solvent 4, ethyl acetate saturated with water. The R_f values of the PE trinitrate metabolites in these solvents are given in Table 1. The biles and

TABLE 1. THIN-LAYER CHROMATOGRAPHY OF PE TRINITRATE AND ITS METABOLITES

Compound*	R_f in solvent†			
	1	2	3	4
PE	0·30	0·54	0·0	0·0
PE mononitrate	0·60	0·81	0·05	0·35
PE dinitrate	0·75	0·92	0·34	0·70
PE trinitrate	0·80	0·97	0·65	0·80
PE mononitrate glucuronide	0·15	0·22	0·0	0·0
PE dinitrate glucuronide	0·15	0·49	0·0	0·0
PE trinitrate glucuronide	0·15	0·67	0·0	0·0

* PE glucuronide was not observed.
† Solvent 1: *n*-butanol–conc. NH_4OH–water (4 : 1 : 3, upper layer).
Solvent 2: *n*-butanol–acetic acid–ether–water (6 : 3 : 9 : 1).
Solvent 3: toluene–ethyl acetate (1 : 1).
Solvent 4: ethyl acetate saturated with water.

urines from the rats treated with PE were submitted to TLC as well as co-chromatography in the above four solvents in addition to solvent 5, *n*-butanol–acetic acid–ether–water (9 : 6 : 3 : 1) and solvent 6, ethanol–water (9 : 1). In these latter solvents PE migrates with R_f values of 0·54 and 0·70 respectively.

Identification of metabolites. The identification of PE trinitrate, PE dinitrate, PE mononitrate and PE by TLC has been documented.[2,4,5] The TLC of bile samples from PE trinitrate treated rats in solvents 1, 3 and 4 showed only a single radioactive peak which did not correspond to any of these four compounds. In solvent 2 this peak was resolved into three distinct components at R_f values of 0·22 ± 0·06,

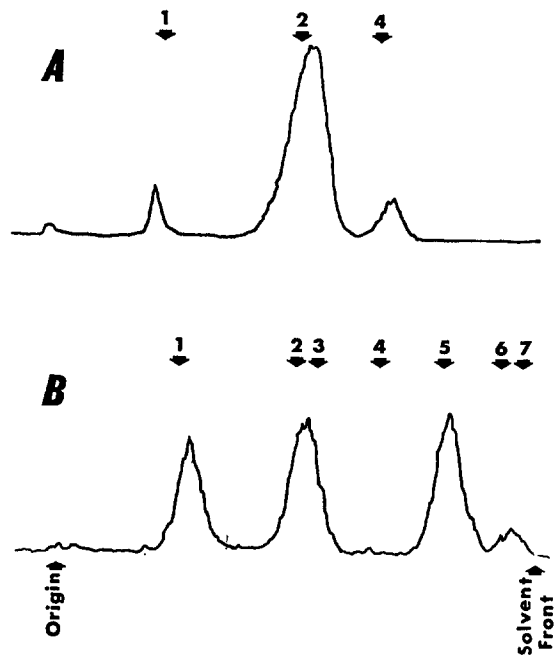

FIG. 1. Representative TLC radioscans of PE trinitrate metabolites in bile (A) and urine (B) samples developed in solvent 2 (*n*-butanol–acetic acid–ether–water, 6 : 3 : 9 : 1) showing the expected metabolite migrations: (1) PE mononitrate glucuronide; (2) PE dinitrate glucuronide; (3) PE; (4) PE trinitrate glucuronide; (5) PE mononitrate; (6) PE dinitrate; and (7) PE trinitrate.

0·49 ± 0·08 and 0·67 ± 0·07 (Fig. 1). Preliminary identification of these compounds as glucuronides was obtained by reaction with β-glucuronidase. Aliquots (20 μl) of the bile were incubated at 37° for 16 hr with a mixture of 2 M, pH 5·0 acetate buffer and 0·2 ml Ketodase (1000 units of β-glucuronidase). The incubation mixtures were submitted to TLC in solvents 1 and 3 to determine the proportions of aglycones produced. These proportions were compared with those of the original glucuronides observed in the bile. Control incubations in the absence of β-glucuronidase showed negligible conversions with the same bile samples.

The identities of the three glucuronide peaks obtained from the bile samples in solvent 2 (Fig. 1) were confirmed by scraping the radioactive areas from the plates and incubating the Silica gel overnight at 37° with 0·5 ml of Ketodase (2500 units of β-glucuronidase) and 0·5 ml of 2·0 M, pH 5·0 acetate buffer. Each incubation mixture was then saturated with ammonium sulfate and extracted three times with 1 ml portions of ethanol–ether (1 : 1) to extract all of the radioactivity. The extracts were concentrated for TLC in solvent 1 to determine the extent of conversion and solvents 3 and 4 to identify the aglycones. Some of the peaks observed on chromatograms of bile samples developed in solvent 2 contained insufficient radioactivity for satisfactory evaluation in three solvents after glucuronidase treatment. In these cases the corresponding peaks from several TLC plates were pooled by elution from the plates with

methanol and redeveloped in solvent 2 to obtain larger peaks with reduced contamination from adjoining peaks.

The identification of the PE trinitrate metabolites in the urines was more complex than the bile because the urine contained unconjugated as well as conjugated metabolites. TLC in solvent 2 separated most of the metabolites (Fig. 1) but did not completely separate PE dinitrate glucuronide from PE and PE dinitrate from PE trinitrate. These two pairs of metabolites were evaluated by also using solvent 1 which completely separated PE from all of the other metabolites and solvent 3 which completely separated PE dinitrate from PE trinitrate.

The identifications of the glucuronides of the three nitrates in the urines were performed in a manner similar to the procedure used for the bile, but the greater dilution and the presence of aglycones required preliminary concentration and isolation of the glucuronides. All of the separate 24-hr urine collections were concentrated by exhaustive extraction of the radioactive compounds with ethanol–ether (1 : 1) after ammonium sulfate saturation. The glucuronides were isolated from the other radioactive compounds by TLC in solvent 1 (cf. R_f values in Table 1). After eluting the glucuronide mixtures with methanol, the extracts were concentrated and resolved by TLC in solvent 2. The glucuronide areas so separated were scraped separately for glucuronidase treatment and rechromatography in solvents 1, 3 and 4 to confirm their identities.

RESULTS

Bile flow. Maximum bile flow rates were observed from 90 to 120 min after bile duct cannulation. For the treated rats, this period was 30–90 min after drug administration. The mean maximum rates of bile flow were 14·4 ± 2·9 mg/min for the controls (Group A), 17·2 ± 0·7 mg/min for the intravenous PE trinitrate (Group B), 15·7 ± 1·1 mg/min for the oral PE trinitrate (Group G) and 15·3 ± 1·8 mg/min for the intravenous PE (Group I) rats. These values do not support a statistically significant choleretic effect even from intravenously administered PE trinitrate because there was rather wide animal-to-animal variation. The mean maximum bile flow rate for all groups corresponded to 47 mg/min/kg, a figure which agrees reasonably well with reported values of 65,[6] 68,[7] and 80[8] obtained from temperature controlled animals, and 55[6] obtained without temperature control.

At 24 hr, the mean flow rates had decreased to 35–48 per cent of the peak values. The 24-hr mean flow rates were 6·1 ± 1·1 mg/min for Group B, 7·6 ± 1·6 mg/min for Group G and 6·6 ± 0·2 mg/min for Group I. These values correspond to a mean of 19 mg/min/kg and are in agreement with a steady-state level after several days of 20 μl/min/kg reported for protein-depleted animals.[3]

Bile radioactivity. As shown in Fig. 2, radioactivity appeared in the bile almost immediately after the administration of either ^{14}C-PE trinitrate or ^{14}C-PE (Groups B, G and I). The rate of ^{14}C excretion reached a maximum within 30 min (Table 2). However, there was a vast difference in the amounts of ^{14}C which entered the bile from ^{14}C-PE trinitrate and ^{14}C-PE. After 24 hr only 0·8% of the PE ^{14}C had been excreted into the bile, whereas approximately 60% of the PE trinitrate ^{14}C was eliminated.

The later time and lower concentration for the maximum bile ^{14}C with the oral dose of ^{14}C-PE trinitrate were not significantly different from the intravenous dose,

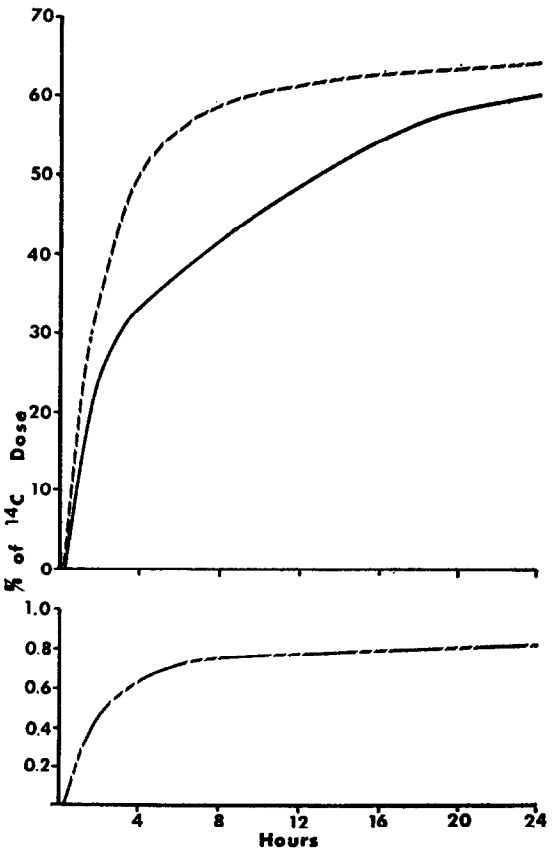

FIG. 2. Cumulative biliary ^{14}C excretion after administration of PE trinitrate 10 mg/kg, i.v., - - - - - and p.o. —— and PE, 10 mg/kg, i.v. — - - —.

although the combined effect produced a significant ($P < 0{\cdot}02$) difference in the total ^{14}C excreted within the first hour (Table 2). The rate of biliary ^{14}C excretion from the orally dosed rats was 63 per cent of that from the intravenously dosed during the first 4 hr post-administration. The rates were approximately equal in the 4–8-hr period, but in the 8–24-hr period three times as much ^{14}C was excreted after the oral as from the intravenous dose. The lack of significance in the difference between the two groups of the total ^{14}C excreted in 24 hr is consistent with the finding of relatively small amounts of radioactivity remaining in the gastrointestinal tracts of the orally dosed bile fistula rats Nos. 3, 4 and 5 (Group G). The amounts found in the three gastrointestinal tracts were 0·9 per cent, 4·0 per cent and 9·5 per cent of the dose, indicating almost complete absorption.

The intravenous administration of an extremely low dose (0·05 mg/kg) of PE trinitrate to one group of rats (Group F) produced a 0–4 hr cumulative biliary excretion (48·6 $\pm$ 5·3 per cent) which was essentially identical to that from the higher dose used in the remainder of this study (50·7 $\pm$ 3·8 per cent). The identical recovery suggests

TABLE 2. BILIARY ^{14}C EXCRETION BY RATS AFTER ^{14}C-DRUG (10 mg/kg) ADMINISTRATION

Drug and route	Peak bile ^{14}C		Per cent of ^{14}C excreted in bile			
	Time (min)*	%/0·1 ml	0–1 hr	0–2 hr	0–4 hr	0–24 hr
PE, i.v.	24 ± 13	0·08 ± 0·03	0·26 ± 0·09	0·47 ± 0·09	0·64 ± 0·07	0·82 ± 0·03
PE trinitrate, i.v.	23·0 ± 3·4	3·1 ± 0·2	19·6 ± 2·0	36·8 ± 2·8	50·7 ± 3·8	64·3 ± 4·4
PE trinitrate, p.o.	31·4 ± 3·9	2·1 ± 0·3	10·4 ± 1·9	21·2 ± 3·4	30·1 ± 3·9	53·8 ± 4·2
P (i.v. vs. p.o.)	NS†	NS	0·02	0·02	0·02	NS

* At bile duct.
† NS = $P > 0·05$.

TABLE 3. RADIOACTIVE GLUCURONIDES IN BILE SAMPLES FROM RATS TREATED WITH 10 mg/kg PE TRINITRATE

Sample time (min)	Glucuronide (%)* of bile ^{14}C					
	PE mononitrate		PE dinitrate		PE trinitrate	
	i.v.	p.o.	i.v.	p.o.	i.v.	p.o.
27·5	0·3 ± 0·2	0·0†	69·1 ± 3·3	68·0 ± 3·1	30·5 ± 3·4	32·0 ± 3·1
47·5	1·5 ± 0·1	0·6 ± 0·6	73·5 ± 2·2	72·7 ± 1·3	24·7 ± 2·4	26·7 ± 1·9
72·5	2·0 ± 0·6	1·3 ± 0·7	(79·3 ± 1·3)‡	(71·3 ± 2·0)‡	(18·7 ± 1·9)‡	(27·4 ± 1·4)‡
117·5	4·4 ± 0·4	6·0 ± 1·0	(81·1 ± 1·3)‡	(66·9 ± 3·5)‡	(14·5 ± 1·7)‡	(27·1 ± 3·2)‡
165	5·4 ± 0·7	5·4 ± 0·6	(81·9 ± 1·1)‡	(62·3 ± 2·8)‡	(12·6 ± 0·6)‡	(31·4 ± 3·4)‡
225	(6·6 ± 0·4)‡	(4·7 ± 0·5)‡	(82·4 ± 0·3)‡	(62·8 ± 3·9)‡	(10·9 ± 0·5)‡	(32·5 ± 3·9)‡
285	6·1 ± 0·5	5·1 ± 1·7	(82·9 ± 1·3)‡	(60·8 ± 1·9)‡	(10·2 ± 1·5)‡	(34·1 ± 3·6)‡
1440	4·2†	7·9 ± 2·9	87·9†	78·2 ± 5·1	7·9†	13·9 ± 2·6

* Mean ± S.E.
† Single value.
‡ Significant difference ($P < 0·05$) between i.v. and p.o. administration.

that the kinetics of the processes responsible for the appearance of radioactivity in the bile are not dose dependent up to the 10 mg/kg PE trinitrate dose level.

Metabolites in bile. The examination of selected bile samples from the bile fistula rats dosed with ^{14}C-PE trinitrate showed the glucuronides of PE mononitrate, PE dinitrate and PE trinitrate as the only radioactive compounds (Table 3). The relative amounts of the three glucuronides excreted into the bile in the first few hours remained essentially constant. From the intravenous dose these amounts were 3·8, 79 and 17 per cent for the glucuronides of PE mononitrate, PE dinitrate and PE trinitrate respectively. After oral dosing the corresponding values were 3·3, 67 and 30 per cent. It is noteworthy that a substantial proportion of PE trinitrate glucuronide was excreted into the bile 24 hr after drug administration by both routes.

Although the proportions of the glucuronides of PE dinitrate and PE trinitrate in the bile differed with the two routes of administration, the entire difference in biliary ^{14}C excretion may be attributed to PE dinitrate glucuronide (Table 4). The

TABLE 4. BILIARY EXCRETION OF GLUCURONIDES IN 0–4 hr INTERVAL

Drug route	Per cent ± S.E. of ^{14}C dose in bile glucuronides*			
	PE mononitrate	PE dinitrate	PE trinitrate	Total
i.v.	1·91 ± 0·20	42·7 ± 2·6	9·3 ± 1·1	53·6 ± 4·0
p.o.	1·06 ± 0·27	23·3 ± 3·7	9·6 ± 1·0	34·1 ± 4·9
P	NS†	0·02	NS	0·05

* Obtained from 3 rats in each group by numerical integration over 0–4 hr at intervals of TLC assay.
† NS = $P > 0·05$.

values from Table 3 were applied to the rate of ^{14}C excretion at the given times to obtain the total 0–4 hr biliary excretion of the metabolites by trapezoidal integration. Over the time interval examined 43 per cent of the intravenous dose and 23 per cent of the oral dose were excreted into the bile as PE dinitrate glucuronide, whereas in both cases 9 per cent of the dose was excreted as PE trinitrate glucuronide. The validity of the integration is supported by the agreement of the calculated total 0–4 hr biliary excretion (Table 4) with the observed values (Table 2) for the same interval.

The radioactivity in the bile from the rats treated with ^{14}C-PE consisted only of PE. No evidence could be found for glucuronide formation. No difference was found between the TLC behavior of synthetic PE and the radioactivity in the bile. No minor peaks were observed on the chromatograms.

Urine radioactivity. Table 5 shows the total 0–24 hr urinary ^{14}C excretion by the intact and bile fistula rats treated with ^{14}C-PE trinitrate by different routes. The route of drug administration had no significant effect upon the total ^{14}C excretion by either the intact or the bile fistula animals. However, there was a highly significant

TABLE 5. DRUG METABOLITES IN URINE COLLECTED FOR 24 hr AFTER ^{14}C-PE TRINITRATE (10 mg/kg) ADMINISTRATION*

Rat group	Per cent dose	Per cent urine ^{14}C as free aglycones				Per cent urine ^{14}C as glucuronides		
	^{14}C in urine	PE	PE mononitrate	PE dinitrate	PE trinitrate	PE mononitrate	PE dinitrate	PE trinitrate
Bile fistula, i.v.	10·9 ± 2·3	7·0 ± 1·8	28·8 ± 3·0	6·1 ± 1·1	0·1 ± 0·1	25·1 ± 1·2	32·3 ± 6·0	1·2 ± 1·2
Intact, i.v.	37·0 ± 6·2	13·5 ± 3·4	28·7 ± 3·5	8·8 ± 2·6	0·4 ± 0·2	21·0 ± 1·8	27·1 ± 3·5	< 0·1
Bile fistula, p.o.	14·6 ± 1·5	5·8 ± 1·9	29·8 ± 7·2	3·2 ± 0·4	<0·1	19·7 ± 0·9	37·7 ± 7·5	3·7 ± 0·3
Intact, p.o.	25·2 ± 4·9	12·7 ± 2·0	31·2 ± 6·0	10·9 ± 1·9	<0·1	24·0 ± 3·5	20·9 ± 6·0	<0·1

* Mean per cent ± S.E. for 3 animals.

difference ($P < 0.005$) in urinary ^{14}C excretion between the bile fistula rats (12·7 ± 1·5 per cent) and the intact (31·1 ± 4·4 per cent). This difference is clearly indicative of the intestinal reabsorption required for enterohepatic circulation.

Metabolites in urine. The urinary radioactivity from the ^{14}C-PE-treated rats consisted entirely of PE. As with the bile, no difference was found between the TLC behavior of synthetic PE and the radioactivity in the urine.

The metabolites found in the 0–24 hr urines of the PE trinitrate-treated rats are given in Table 5. The major urinary metabolites were PE mononitrate and the glucuronides of PE mononitrate and PE dinitrate. These latter two compounds accounted for 57 per cent of the urinary radioactivity of the bile fistula animals and 46 per cent of that of the intact animals. All four groups excreted approximately 30 per cent of the urinary ^{14}C as PE mononitrate. Smaller amounts of PE (6–14 per cent) and PE dinitrate (3–11 per cent) were also found. Trace amounts of PE trinitrate and PE trinitrate glucuronide were found in the urines of many animals, but PE glucuronide was not observed.

The compositions of the 0–24 hr urine collections after ^{14}C-PE trinitrate administration, expressed as per cent of the ^{14}C dose, were evaluated as 2 × 2 Latin Square (Table 6). The quantities of PE trinitrate and its glucuronide were too small for

TABLE 6. STATISTICAL EVALUATION OF URINARY METABOLITES

Group	Mean % of dose ^{14}C*				
	Aglycones			Glucuronides	
	PE	PE mononitrate	PE dinitrate	PE mononitrate	PE dinitrate
Intact, i.v.	4·50	10·2	3·00	7·5	9·6
Bile fistula, i.v.	0·67	2·9	0·57	2·6	3·2
Intact, p.o.	3·01	7·3	2·56	5·7	5·5
Bile fistula, p.o.	0·75	4·1	0·45	2·8	5·2
i.v.	1·74	5·5	1·31	4·4	5·5
p.o.	1·50	5·5	1·07	4·0	5·3
Intact	3·68	8·6	2·77	6·6	7·2
Bile fistula	0·71	3·5	0·51	2·7	4·1
P (i.v. vs. p.o.)	NS†	NS	NS	NS	NS
P (intact vs. bile fistula)	< 0·001	0·01	< 0·001	0·002	±‡

* Means evaluated as logarithms.
† NS = $P > 0.1$.
‡ ± = $0.1 > P > 0.05$.

proper evaluation. It is obvious from Table 6 that the route of administration had no effect on the 0–24 hr urine composition. The decrease of the total urinary ^{14}C excreted by the bile fistula rats is reflected not only by decreases of the glucuronides but also by highly significant decreases of the aglycones. The intact rat to bile fistula rat ratio was 1·9 ($P < 0.02$) for the glucuronides and 3·2 ($P < 0.0005$) for the aglycones. The P values assigned to these ratios refer to the significance of their difference from

unity. The bile fistula rats excreted 43% less PE dinitrate glucuronide and 59% less PE mononitrate glucuronide than the intact rats. The decreases in aglycone excretion were 87% PE dinitrate, 59% PE mononitrate and 80% PE.

Blood radioactivity. The blood levels found in rats at short intervals after intravenous ^{14}C-PE (Group J) or ^{14}C-PE trinitrate (Groups D and E) administration are given in Table 7. The PE blood levels decreased in a manner which indicates a fit to a

TABLE 7. BLOOD RADIOACTIVITY AFTER 10 mg/kg (i.v.) ^{14}C-DRUG ADMINISTRATION

Min	% ^{14}C dose/ml blood after	
	PE	PE trinitrate*
5	0·58 ± 0·05	0·25 ± 0·01
20	0·32 ± 0·03	0·20 ± 0·01
35	0·21 ± 0·02	0·20 ± 0·01
50	0·16 ± 0·01	0·20 ± 0·01
65	0·13 ± 0·01	0·21 ± 0·01

* Combined mean of intact and ligated animals.

multicompartment pharmacokinetic model (minimum of two compartments). However, the interval of observations was too short to evaluate the curve on the basis of a given model. The blood levels observed for the PE trinitrate dose remained essentially constant over the time interval studied and the same values were obtained from the ligated animals (Group E) as from the intact animals (Group D). The value of a 0·2% dose/ml of blood indicates a distribution volume of the order of 15 times blood volume.

From 20–65 min after ^{14}C-PE administration, the ratio of bile ^{14}C to blood ^{14}C was 1·9 ± 0·2, indicating mainly passive transfer into the bile. The situation was greatly different during the same interval after ^{14}C-PE trinitrate dosing; the ratio of bile to blood radioactivity ranged from 95–143, strongly indicating an active transport mechanism.

DISCUSSION

An especially interesting outcome of this study was discovering new metabolites of 3 pentaerythritol nitrates. These compounds were identified as glucuronides of PE mononitrate, PE dinitrate and PE trinitrate. The solvent systems used earlier for thin-layer chromatography did not separate these glucuronides from PE. The urinary excretion attributed to PE after PE trinitrate administration to rats[2] corresponds to the sum of the PE nitrate glucuronides and PE found in the present investigation. Glucuronide metabolites of polyhydric compounds were recognized as early as 1901[9] and were studied extensively by Gessner *et al.*,[10] but Dietz[11] was the first to report a glucuronide of an organic nitrate, namely of a 1-chloro-2,3-propanediol mononitrate. More recently, Reed *et al.*[12] have reported the glucuronide of endo-isosorbide mononitrate. It is interesting to note that isosorbide was found as a

glucuronide in dog urine[12] but that PE glucuronide could not be detected in rat urine or bile. It should be added that PE administration to mice[13] and dogs[14] as well as to rats led to no detectable PE glucuronide. In the light of these observations, one may question whether isosorbide glucuronide was formed from isosorbide or from isosorbide mononitrate glucuronide.

In view of the widespread occurrence of β-glucuronidase in the rat[15] and the susceptibility of PE nitrate glucuronides to this enzyme demonstrated in the present communication, the biotransformation of PE trinitrate in the rat is considered to include the following reversible and irreversible reactions, shown in Fig. 3.

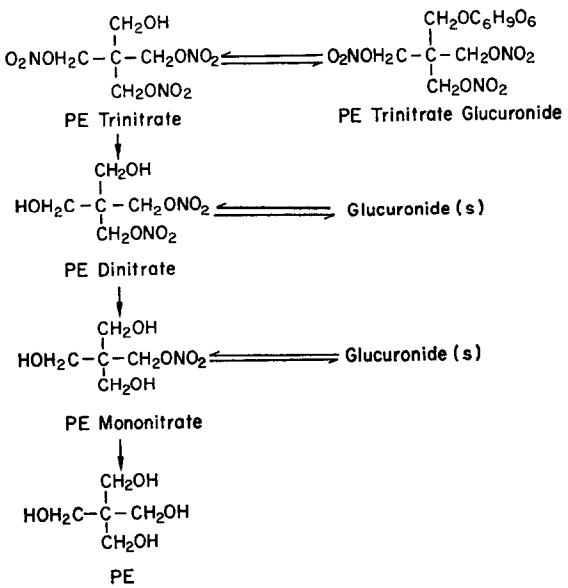

FIG. 3. Pathways of PE trinitrate biotransformation.

The structure of PE trinitrate glucuronide is fully defined because the parent compound can accommodate only one glucuronic acid residue. The number of glucuronic residues attached to PE mononitrate and PE dinitrate is unknown. It has been well documented[16] that glucuronides having molecular weights above 400 have a propensity for excretion in the bile. The conjugation of PE nitrates with glucuronic acid brings their mol. wt. into this range. PE trinitrate glucuronide has a mol. wt. of 447 and the molecular weights of the monoglucuronides of PE dinitrate and PE mononitrate are 402 and 357 respectively. Thus, the biliary excretion of these metabolites correlates with the general molecular weight pattern[16] and with the observed active transport into the bile which is typical of class B acids as defined by Brauer[17] and Stowe and Plaa.[18]

Collecting bile from rats treated with ^{14}C-PE trinitrate caused a 60 per cent reduction of the quantity of radioactivity which normally was excreted in the urine (Table 5). This finding indicated that the biliary drug glucuronides were reabsorbed

extensively from the intestine of the intact animals. It seems probable that the glucuronides were not absorbed intact,[16] but that they were hydrolyzed in the intestine by β-glucuronidase originating from the bile[16] or from the intestinal flora.[19] This sequence seems very probable in view of the observed ease of hydrolysis of PE nitrate glucuronides and the high aglycone content of the urine from intact rats (Table 5). Accordingly, we consider that the data are best explained by the enterohepatic circulation of PE trinitrate metabolites. Judging from the observation that the oral and intravenous administration of PE trinitrate allowed the transfer of approximately the same quantity of PE trinitrate glucuronide into the bile, one may expect both routes of drug administration to produce long-lasting pharmacological effects in the rat.

Addendum—After this paper was submitted, a pertinent report by S. F. SISENWINE and H. W. RUELIUS appeared in *J. Pharmac. exp. Ther.* **176,** 296 (1971). On the basis of products excreted following the administration of [^{14}C]isosorbide dinitrate and [^{14}C]isosorbide to dogs, these authors concluded that isosorbide glucuronide was formed exclusively from 5-isosorbide mononitrate glucuronide by denitration.

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