Binding of Pentaerythritol Tetranitrate and Its Metabolites by Rat Blood Plasma and Erythrocytes. (30631)

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In the course of studying the metabolism of pentaerythritol tetranitrate (PETN), it became of interest to develop information on the binding of PETN by blood protein, particularly since this point may have bearing upon the long duration of anginal prophylaxis induced by PETN. This matter was investigated *in vitro* by dialyzing C^{14}-PETN with rat blood plasma for various periods at two pH levels. Parallel studies were conducted with rat erythrocytes since these cells seemed especially capable of effecting the degradation of PETN(1). Both components of each dialysis system were assayed quantitatively for total activity as well as for PETN and each metabolite. The latter assays were performed by thin layer and radioscanning methods described previously(2).

Materials and methods. C^{14}-PETN, labeled at carbon atoms 1 and 2, was synthesized from acetaldehyde *via* pentaerythritol (PE). In order to minimize the explosion hazard, the PETN (m.pt., 140-141.5°) was dissolved in ether and then mixed with C.P. lactose (7 times the weight of PETN). After the solvent was removed, the radioactivity of the lactose-C^{14}-PETN mixture was 0.59 millicurie/g.

Blood from female Wistar rats was used to prepare plasma and red blood cells. The cells were washed 4 times with 0.2 M phosphate buffer at pH 7.4 and suspended in this buffer to the original blood volume.

Aliquots of a suspension of C^{14}-PETN in propylene glycol were added to 10.0 ml aliquots of the plasma and the erythrocyte suspension contained in Visking sausage casing to produce concentrations of approximately 50 μg of PETN per ml. Aliquots of each mixture were dialyzed against 50.0 ml of 0.2 M phosphate at pH 5.7 and 7.4. Separate dialyses were run at 37° with mild agitation for 4, 6, 18, 24, 48 and 72 hours. At the conclusion of each dialysis experiment, aliquots of both components of the system were counted for total radioactivity by scintillation spectometry in a Packard TriCarb operating at 12°. Depending upon the C^{14} content, the buffer component was either counted directly or first diluted with water. Aliquots of the plasma or erythrocyte components were diluted with distilled water (50 or 100 ml). In each instance, 1.0 ml of test material was mixed with 20 ml of scintillation solution consisting of 7.0 g of 2,5-diphenyloxazole, 0.3 g of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene and 100 g of naphthalene in 1.0 l of freshly distilled dioxane. An internal standard was used to evaluate the quenching characteristics of each sample. The remainders of the plasma and erythrocyte components were diluted to 50 ml with freshly distilled dioxane and frozen. The dialysates were also frozen (without further dilution) pending assay for the PETN degradation products.

The dialyzed erythrocyte components were filtered prior to further concentration. The dialyzed plasma components and all of the dialysates were concentrated without prior filtration. An aliquot (10.0 ml) of each fraction was diluted to 50 ml with methanol. After separation of precipitated material by centrifugation, the supernatant solutions were reduced to a volume of about 1 ml by an air stream at room temperature. The residual material was then diluted to 20 ml with methanol resulting in the further separation of insoluble material. The supernatant solution was evaporated once more to dryness and the residue was triturated 5 times with 1.0 ml portions of methanol. The methanol was concentrated to a volume less than 0.5 ml, diluted to 2.0 ml with a mixture of methanol and ethyl acetate (1:1, v/v) and centrifuged to remove additional insoluble material which separated. The solution was finally concentrated to a volume of 0.1-0.2 ml for thin layer chromatography.

Portions (20-200 μl) of the final concentrates containing C^{14} corresponding to approximately 10 μg of the original C^{14}-PETN were spotted for thin layer chromatography on glass plates (2″ × 8″) coated with silica gel G. The chromatograms were developed in toluene-ethyl acetate-1-butanol-water (10:5:2:2, v/v/v/v, upper phase). This solvent system separates PETN and the 4 degradation products with the following Rf values: PE, 0.00; PE-mononitrate, 0.16; PE-dinitrate, 0.45; PE-trinitrate, 0.60; PETN, 0.77.

TABLE I. Distribution of C^{14}-PETN and Degradation Products During Dialysis from Rat Blood Plasma.

Component	Buffer (pH)	Time (hr)	% radioactivity					
			PE	PE-mono-nitrate	PE-di-nitrate	PE-tri-nitrate	PETN	Total
Dialyzed plasma	7.4	4	—	—	—	6.1	79.7	85.8
		18	—	—	1.2	6.1	64.9	72.2
		24	—	—	1.8	7.2	58.3	67.3
		48	—	—	1.8	6.6	32.1	40.5
		72	1.4	2.9	8.1	3.4	21.5	37.3
Plasma dialysate	7.4	4	—	—	—	4.5	9.7	14.2
		18	—	—	2.3	12.3	13.2	27.8
		24	—	—	2.5	14.6	15.6	32.7
		48	—	—	8.3	36.9	14.3	59.5
		72	5.8	14.0	30.4	11.3	1.2	62.7
Dialyzed plasma	5.7	4	—	—	—	3.7	81.3	85.0
		18	—	—	1.0	5.7	53.0	59.7
		48	1.7	.8	3.8	9.2	36.5	52.0
		72	1.0	.5	4.3	12.6	28.8	47.2
Plasma dialysate	5.7	4	—	—	—	2.0	13.0	15.0
		18	.8	—	5.0	18.1	16.4	40.3
		48	2.2	—	10.7	25.7	9.4	48.0
		72	1.3	.6	11.2	25.4	14.3	52.8

TABLE II. Distribution of C^{14}-PETN and Degradation Products During Dialysis from Rat Blood Erythrocytes.

Component	Buffer (pH)	Time (hr)	% radioactivity					
			PE	PE-mono-nitrate	PE-di-nitrate	PE-tri-nitrate	PETN	Total
Dialyzed erythrocytes	7.4	4	1.3	1.9	7.6	25.2	54.2	90.2
		6	1.9	2.8	6.7	17.1	43.8	72.3
		18	3.2	7.4	8.4	29.6	21.1	69.7
		24	2.3	5.3	7.8	23.2	15.1	53.7
		48	3.2	4.8	3.4	15.1	15.9	42.4
		72	4.2	13.3	4.9	8.4	10.7	41.5
Erythrocyte dialysate	7.4	4	.4	.7	2.8	3.9	2.0	9.8
		6	1.5	2.5	8.2	10.5	5.0	27.7
		18	2.4	9.6	13.1	4.0	1.2	30.3
		24	4.4	14.9	17.5	7.0	2.5	46.3
		48	11.2	27.9	14.0	3.1	1.4	57.6
		72	10.4	34.2	13.2	.7	0	58.5
Dialyzed erythrocytes	5.7	4	trace	trace	1.5	17.6	62.5	81.6
		6	.8	"	2.8	15.3	61.5	80.4
		18	.9	.9	5.3	23.0	32.2	62.3
		48	3.6	6.5	15.1	10.9	21.0	57.1
		72	4.5	8.8	5.3	2.2	6.3	27.1
Erythrocyte dialysate	5.7	4	.2	.2	1.3	7.9	8.8	18.4
		6	.2	.2	1.9	8.6	8.7	19.6
		18	.7	1.1	9.8	20.2	5.9	37.7
		48	1.2	12.7	27.8	1.2	trace	42.9
		72	3.4	28.5	30.9	6.8	3.3	72.9

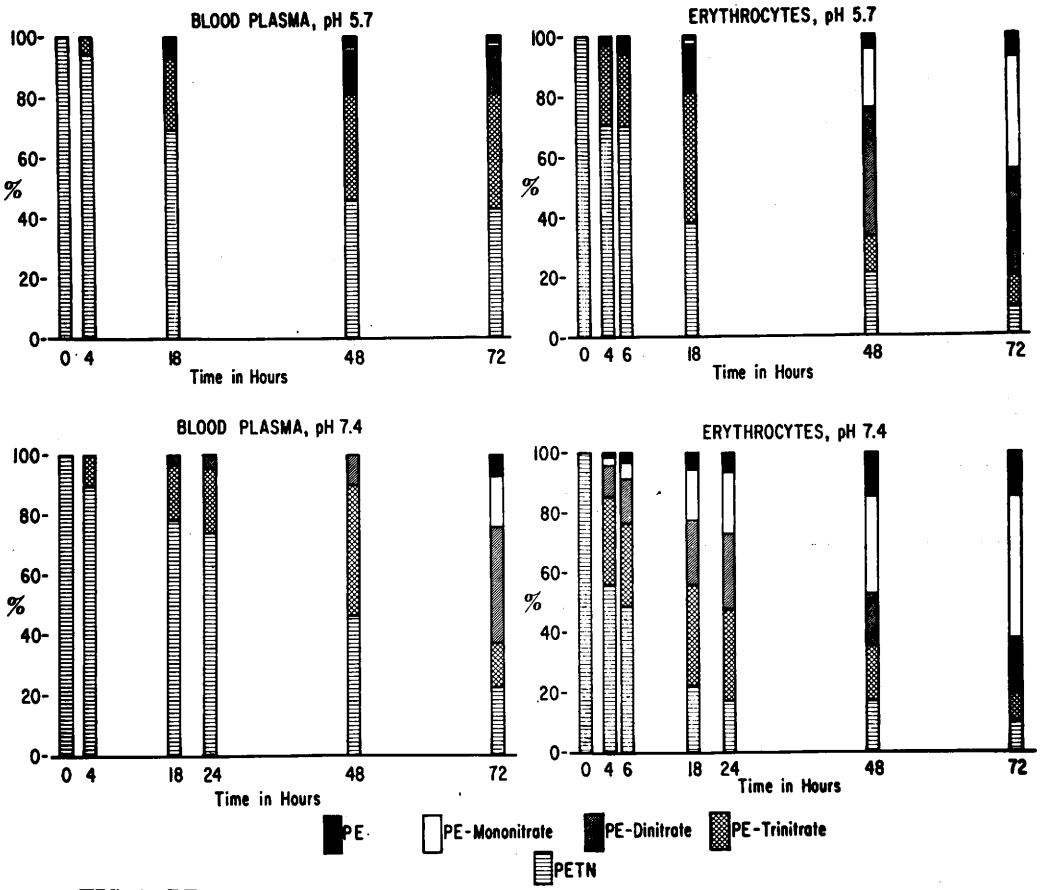

FIG. 1. PETN degradation during dialyses with rat blood plasma and erythrocytes at pH 5.7 and 7.4.

The chromatograms were scanned for radioactivity with a Packard Model 7200 Radiochromatogram Scanner. The area under each curve was determined with a Keuffel and Esser compensating polar planimeter.

Results. Table I shows the distribution of the degradation products of PETN after dialysis from rat blood plasma for the given time intervals. Table II shows the distribution of compounds present after dialysis from rat blood erythrocytes. The over-all course of degradation in the 2 media is shown in Fig. 1. The degradation of the PETN by the plasma occurred at a linear velocity with the lower nitrates competing successfully with the PETN for degradation. The degradation by the erythrocytes was non-linear and more extensive than by the plasma. The trinitrate and dinitrate competed successfully with the PETN for degradation with a consequent increase in mononitrate concentration. Both in the case of the plasma and of the erythrocytes, the degradation proceeded more slowly at pH 5.7 than at 7.4.

Tables III and IV show the apparent binding of PETN and its degradation products by rat blood plasma and erythrocytes. The precision of these data suffers from cumulative errors of counting and radioscanning, and for this reason were rounded off to the nearest 5%. It is evident from Tables I and II that the quantities of some components of the dialysis mixtures were too small to assess the degree of binding; in Tables III and IV, these situations are indicated by the term insufficient sample. The binding of PETN was of high order in all cases. The binding of the trinitrate was high to the erythrocytes and low to the plasma. The lower nitrates did not appear to be bound

TABLE III. Apparent Binding of C^{14}-PETN and Degradation Products to Rat Blood Plasma.

Time (hr)	Buffer (pH)	Apparent % bound*					
		PE	PE-mononitrate	PE-dinitrate	PE-trinitrate	PETN	Total
4	7.4	—	—	—	50	85	83
18	—	—	—	I.S.	20	80	67
24	—	—	—	I.S.	20	75	61
48	—	—	—	0	0	65	29
72	—	I.S.	0	5	5	95	25
4	5.7	—	—	—	60	85	82
18	—	I.S.	—	0	10	70	52
48	—	I.S.	I.S.	10	10	75	42
72	—	I.S.	I.S.	10	20	60	37

* Values for individual components expressed to nearest 5%. I.S. = insufficient sample.

TABLE IV. Apparent Binding of C^{14}-PETN and Degradation Products to Rat Blood Erythrocytes.

Time (hr)	Buffer (pH)	Apparent % bound*					
		PE	PE-mononitrate	PE-dinitrate	PE-trinitrate	PETN	Total
4	7.4	I.S.	I.S.	70	85	95	88
6	—	I.S.	45	35	55	90	67
18	—	50	30	25	85	95	64
24	—	20	10	15	70	80	44
48	—	5	0	5	80	90	31
72	—	15	15	15	90	100	30
4	5.7	I.S.	I.S.	I.S.	I.S.	85	78
6	—	I.S.	I.S.	50	55	85	76
18	—	I.S.	I.S.	20	45	80	55
48	—	70	20	20	90	100	48
72	—	50	10	0	10	60	12

* Values for individual components expressed to nearest 5%. I.S. = insufficient sample.

to any significant extent.

Discussion. The present binding study was complicated by the enzymatic cleavage of PETN and the other nitrates. Although the complication was recognized from previous study(1), the experimental design was employed because it permitted the development of information on the binding of the other nitrates of PE. These nitrates have been synthesized(3), but were not available for study in this laboratory. The main problem was that the sustained enzymatic degradation of the organic nitrates made it difficult to establish equilibria between the dialyzed suspensions and their dialysates. Another factor was that the concentrations of the compounds changed with time as they were being degraded and/or formed. Changes in concentration present a problem since the binding of drugs by blood proteins frequently follows Freundlich's adsorption isotherm(4) which means that the percentage of drug bound varies inversely with the drug concentration when the protein concentration is constant. Despite these considerations, the experimental data indicate clearly that PETN was strongly held by plasma and erythrocyte components. It is also evident that PE-trinitrate showed a strong affinity for erythrocytes, but not for plasma. There was no significant binding of PE, PE-mononitrate or PE-dinitrate. The long duration of anginal prophylaxis afforded by PETN may be related to its binding property as well as to its greater resistance to enzymatic degradation than nitroglycerine(5).

It appears probable that binding of PETN, presumably by non-enzymatic blood proteins, decreased its availability for enzymatic conversion. This consideration of binding is noteworthy since it is often overlooked in experimental systems intended to assess the relative specificity of a given enzyme for a series of substrates.

Erythrocytes were more effective than

blood plasma in converting the PE nitrates. Crandall(6) attributed the destruction of nitroglycerine by blood to the action of the erythrocytes. PE-mononitrate was attacked at the slowest rate. In general, each lower nitrate appeared to be digested at a rate slower than was its predecessor; if the situation were otherwise, sizeable quantities of the lower nitrates would not have been present. The above-mentioned influence of binding precluded the derivation of definitive information on the order of the enzymatic reactions. Such data are anticipated from enzyme studies with purified systems.

Summary. Rat blood plasma and red cells showed strong affinity for PETN. In both systems, PETN was degraded stepwise to PE-trinitrate, PE-dinitrate, PE-mononitrate and PE. PE-trinitrate was bound extensively by erythrocytes. PE, PE-mononitrate and PE-dinitrate were not bound to a significant degree by plasma or erythrocytes. Erythrocytes were more effective than plasma for the degradation of PETN. PE-mononitrate was the most resistant substrate in the series.

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Thermal Separation of the Synthesis of Papovavirus SV40 Tumor and Virus Antigens.* (30632)

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A specific intranuclear antigen appears in hamster tumors induced by papovavirus SV40, or in hamster or human cells transformed by the virus(1-8). This tumor (T) antigen can be detected by complement-fixation or by immunofluorescence. In cells infected by SV40, synthesis of the same T antigen is induced early in the cytolytic cycle before the appearance of the virus itself(9-11). In the presence of cytosine arabinoside, the synthesis of T antigen is unimpeded, but the production of viral antigen is completely blocked(12).

Further knowledge of this T antigen is needed, particularly of the conditions under which it is synthesized. In the present study it is shown that the syntheses of SV40 tumor (T) and virus (V) antigens proceed at different rates at different temperatures. Furthermore, conditions can be manipulated so that infected cells produce either T or V antigen.

Materials and methods. Monolayer cultures of green monkey kidney (GMK) cells were grown in M-H medium and maintained in M-E medium(13). They were inoculated with SV40 at an input multiplicity of 10 TCD_{50} per cell. After adsorption for 2 hours at 37°C, the cultures were incubated at 23°, 37°, 40°, 42° and 43°. At intervals, the infected cells were collected by scraping them from the bottle with a rubber policeman. They were centrifuged and resuspended in pH 7.3 veronal buffered saline (VBS) to make a 5% suspension. The suspensions were treated for 30 seconds in a Raytheon sonic oscillator model 101 at 10KC, and then were centrifuged at 3000 rpm. The supernatant

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