The Fate of [^{14}C]Thalidomide in the Pregnant Rabbit

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(Received 29 November 1966)

1. The fate of [^{14}C]thalidomide orally administered to pregnant rabbits at the beginning of the sensitive phase of pregnancy has been studied. 2. After the oral administration of [^{14}C]thalidomide on the 192nd hour of pregnancy about 68% of the radioactivity appears in the urine and 22% in the faeces. 3. The urinary ^{14}C is made up as follows (% of dose): thalidomide (2); α -(*o*-carboxybenzamido)glutarimide (16); 2- and 4-phthalimidoglutaramic acids (11); 2-phthalimidoglutaric acid (0.2); 2- and 4-(*o*-carboxybenzamido)glutaramic acids and 2-(*o*-carboxybenzamido)glutaric acid (29). 4. The plasma ^{14}C concentration is maximal at 12 hr. after dosing and the radioactivity persists for more than 58 hr. At 4 hr. the main compound in the plasma is thalidomide, but its concentration steadily declines while the concentration of its hydrolysis products increases. 5. At 12, 24 and 58 hr. after dosing radioactivity is present in the embryo and the maternal tissues examined. The ^{14}C concentration in the embryo is at nearly all times higher than that in the plasma, brain, skeletal muscle and fat but lower than that in the liver and kidney. 6. At 4 hr. after dosing the mother on the tenth day of pregnancy the specific activities of the embryo and the yolk-sac fluid are similar. 7. Thalidomide is found in the embryo together with seven of its hydrolysis products for more than 24 hr. after dosing. The accumulation of radioactivity in the embryo is due to retention of the polar hydrolysis products.

Previous studies from this Laboratory have shown that the embryo of the New Zealand White rabbit is sensitive to the teratogenic effect of thalidomide during a period of 58 hr. extending from the 192nd to 250th hour of pregnancy (Fabro & Smith, 1966). The fate of [^{14}C]thalidomide in the pregnant rabbit during this phase of gestation has therefore been studied.

MATERIALS AND METHODS

Materials. [^{14}C]Thalidomide [($\pm$)-3-([carbonyl- $^{14}\text{C}_1$]-phthalimido)glutarimide; specific activity 0.75 $\mu\text{C}/\text{mg}$; m.p. 270° was synthesized from [carbonyl- $^{14}\text{C}_1$]phthalic anhydride by the method of Beckmann (1962). Thalidomide (m.p. 271°) was a gift from Lilly Research Laboratories Ltd. (Bromborough Port, Cheshire). Phthalic acid, m.p. 219°, was purchased. ($\pm$)-2- and ($\pm$)-4-Phthalimidoglutaramic acids, ($\pm$)-2-phthalimidoglutaric acid, ($\pm$)-4-(*o*-carboxybenzamido)glutaramic acid and ($\pm$)- α -aminoglutarimide were samples previously prepared (Schumacher, Smith & Williams, 1965). ($\pm$)- α -(*o*-Carboxybenzamido)glutarimide, m.p. 268–270°, was prepared from phthalic anhydride and α -aminoglutarimide (Sondheimer & Holley, 1957) by the method of Beckmann (1963).

Animal experiments. Adult New Zealand White does (3–4 kg. body wt.) were mated with bucks of the same breed. Immediately after copulation the female was removed from the cage of the male and the time of mating was taken as

hour zero of the pregnancy. The pregnant animals were allowed free access to food and water, and on the 192nd hour of pregnancy they were each given, by stomach tube, [^{14}C]thalidomide (150 mg./kg.; 5 $\mu\text{C}/\text{kg}$.) suspended in about 20 ml. of water. This dose is teratogenic in this breed of rabbit (Fabro & Smith, 1966).

Four groups of three treated animals were killed by a blow on the head at 4, 12, 24 and 58 hr. respectively after dosing. Samples of maternal tissues including blood were removed, and the urine and faeces of each animal were collected. Blood samples (1–2 ml.) were withdrawn from the ear vein of the appropriate animals at 4, 8, 12, 24 and 58 hr. after dosing and the plasma was obtained by centrifuging the blood at 2000 rev./min. in an MSE Minor centrifuge S17–750 for 10 min.

Embryos free from blood and uterine tissue were isolated as described by Fabro, Schumacher, Smith & Williams (1964). In some experiments the embryo was separated from the yolk-sac fluid by centrifuging at 10000 g for 20 min. at 0° in an MSE high-speed centrifuge (model 17).

Preparation of samples for ^{14}C estimation. Embryos (0.6–1.4 g. wet wt.), maternal plasma (0.2–0.5 ml.) or urine (0.5–1 ml.) were dispersed in sufficient liquid scintillation fluid A contained in a glass counting vial to give a final volume of 20 ml. Scintillation fluid A consisted of dioxan-ethylene glycol-methanol (44:1:5, by vol.) mixture containing naphthalene (6%), 2,5-diphenyloxazole (0.4%), 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.02%) and thixotropic gel powder (Cab-O-Sil, 5%). Samples (5–13 g.) of maternal liver, kidney, brain, skeletal muscle or perirenal

fat were treated with 2N-acetic acid (1ml./g. wet wt. of tissue) and homogenized in 40ml. of dioxan-methanol (1:1, v/v) mixture. This homogenate was diluted to 100ml. with the dioxan-methanol mixture and 1-5ml. samples were taken for ^{14}C determinations. Samples of faeces or of intestine (including contents) were treated with acetic acid (0.1ml./g. wet wt. of material), homogenized in dioxan-methanol as above and then diluted to 2l. with the same solvent. ^{14}C was determined in 1-2ml. samples.

In some experiments, the samples were burned in a Schöniger flask to $^{14}\text{CO}_2$, which was collected in M-Hyamine hydroxide in methanol (10ml.) (Kelly, Peets, Gordon & Buyske, 1961). The Hyamine solution (3ml.) was transferred to glass counting vials containing 17ml. of scintillation fluid B [toluene containing 2,5-diphenyloxazole (0.5%) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.03%)].

^{14}C was counted in a Packard Tri-Carb liquid-scintillation spectrometer (model 3214). Blanks were prepared from the tissues of untreated pregnant does. Counting efficiency was measured by the twin-channel-ratio method (Bush, 1963). For samples with low radioactivity (i.e. counting rate less than three times that of background), the efficiency was measured by the internal-standard method.

Identification of ^{14}C -labelled compounds. Radioactive compounds in the embryo, maternal tissues and excreta were identified by a reverse isotope-dilution technique after a preliminary chromatographic separation. Pooled homogenized embryos (six to ten) were freeze-dried to a yellowish-red powder, which was extracted three times with methanol (10ml.). After centrifuging, the extract was concentrated under reduced pressure at 30° to 2ml. in a rotary evaporator. Samples (0.3-0.5ml.) of the extract were dried as a spot on Whatman no. 1 paper. Over this spot was applied 0.1ml. of a methanolic solution containing 50 μg . of each of the carrier compounds, namely thalidomide, 2- and 4-phthalimidoglutaric acid, 2-phthalimidoglutaric acid, α -(*o*-carboxybenzamido)glutarimide, 2- and 4-(*o*-carboxybenzamido)glutaramic acids, 2-(*o*-carboxybenzamido)glutaric acid and phthalic acid. The chromatograms were developed in two dimensions with the solvent systems of Schumacher *et al.* (1965). After being dried, the chromatograms were examined in ultraviolet light (Chromatolite lamp, peak emission 254m μ) before and after hydrazine treatment as described by Schumacher *et al.* (1965). The compounds were identified by comparing their R_F values

and colour reactions with those of authentic samples (Schumacher *et al.* 1965). Maternal plasma (10ml.) was similarly freeze-dried and treated as above. With urine and faecal extracts, samples (0.1ml.) were applied directly to the paper.

For estimation of ^{14}C , areas of the paper chromatogram, known to contain specific compounds, were cut out and transferred to counting vials containing 20ml. of scintillation fluid B. The rest of the chromatogram was similarly cut into pieces of about 3cm. $\times$ 3cm. and counted. The total ^{14}C on the chromatogram was then estimated, and, after correction for background, the ^{14}C associated with each compound was calculated as a percentage of the total.

RESULTS AND DISCUSSION

The embryo of the New Zealand White rabbit is sensitive to the teratogenic effect of thalidomide for a period of about 58hr., that is from the 192nd to the 250th hour of pregnancy (Fabro & Smith, 1966). If the mother is given an oral dose of the drug of 150mg./kg. at the beginning of this period, then by the end of this time some 90% is eliminated, about 70% in the urine and 20% in the faeces (Table 1).

Table 1. *Distribution of ^{14}C in the excreta and gut of pregnant rabbits receiving [^{14}C]thalidomide orally*

[^{14}C]Thalidomide (150mg./kg.; 5 μC /kg.) suspended in about 20ml. of water was administered orally to six pregnant rabbits at the 192nd hour of pregnancy. ^{14}C was determined in the urine, faeces and gut and its contents at 24hr. in rabbits 1-3 and at 58hr. in rabbits 4-6. Average values are given with the ranges in parentheses.

Material analysed	^{14}C found (% of dose)	
	At 24hr. (rabbits 1-3)	At 58hr. (rabbits 4-6)
Urine	62.7 (60.2-65.3)	67.9 (66.1-70.3)
Faeces	17.0 (13.6-20.0)	21.6 (17.7-24.8)
Gut and contents	8.1 (6.3-9.3)	3.4 (2.4-4.3)
Total	87.8 (84.0-91.3)	92.8 (87.2-96.9)

Table 2. *Radioactive compounds in the urine and gut of rabbits at 24hr. after receiving [^{14}C]thalidomide orally*

Dosage was as in Table 1. The results are for rabbits 1-3 of Table 1; average values and ranges are given. The compounds were separated chromatographically on paper and specific areas of this cut out for counting (see the text).

Compound determined	^{14}C found after 24hr. (% of dose)	
	In urine	In gut and contents
Thalidomide	1.7 (1.1-2.5)	5.6 (4.1-6.2)
α -(<i>o</i> -Carboxybenzamido)glutarimide	15.6 (11.7-21.4)	1.8 (1.3-2.2)
2- and 4-Phthalimidoglutaramic acids	10.6 (8.7-13.1)	0.1 (0.06-0.2)
2-Phthalimidoglutaric acid	< 0.2	< 0.1
2- and 4-(<i>o</i> -Carboxybenzamido)glutaramic acids + 2-(<i>o</i> -carboxybenzamido)glutaric acid	28.8 (26.7-32.1)	0.5 (0.3-0.8)
Total	56.9 (54.0-61.6)	8.0 (5.9-9.4)

Fig. 1. Plasma ¹⁴C concentrations of pregnant New Zealand White rabbits after the oral administration of [¹⁴C]thalidomide (150 mg./kg.; 5 μ C/kg.) on the 192nd hour of pregnancy. Each point is the mean value for three or more animals.

Table 3. Radioactive compounds in the plasma of pregnant rabbits after receiving [¹⁴C]thalidomide orally

Dosage was as in Table 1. Each value quoted is the mean for three animals. —, Not detected.

Compound determined	Plasma concn. (μ g./ml.)		
	At 4hr.	At 12hr.	At 24hr.
Thalidomide	7.6	4.1	1.3
α -(o-Carboxybenzamido)-glutarimide	1.7	11.4	4.3
2- and 4-Phthalimidoglutaramic acids	0.5	2.4	0.8
2-Phthalimidoglutaric acid	—	0.5	0.5
2- and 4-(o-Carboxybenzamido)-glutaramic acids + 2-(o-carboxybenzamido)glutaric acid	—	2.8	1.6

The urinary material contains only 2% of the dose as unchanged thalidomide, the rest being hydrolysis products. The faecal material, however, is largely unchanged thalidomide, with smaller amounts of the hydrolysis products, the main one being α -(o-carboxybenzamido)glutarimide (Table 2). Thalidomide at this dosage is thus fairly well absorbed from the intestine by the pregnant rabbit. The plasma radioactivity, shown in Fig. 1, reaches a maximum at about 12 hr. after dosing and then declines so that by 58 hr. it reaches a value of about 15% of the maximum. The nature of the ¹⁴C in the plasma at various times up to 24 hr. after dosing is shown in Table 3. This Table shows a steady decline in the plasma thalidomide concentration. At 4 hr. the primary hydrolysis products, α -(o-carboxybenzamido)glutarimide and 2- and 4-phthalimidoglutaramic acids, are detectable. By 12 hr. these have increased, especially the first, which reaches a concentration nearly three times that of thalidomide, and now the secondary hydrolysis products, 2-phthalimidoglutaric acid, 2- and 4-(o-carboxybenzamido)glutaramic acids and 2-(o-carboxybenzamido)glutaric acid, are also present. At 24 hr., the plasma concentrations of all the hydrolysis products have declined owing to excretion.

Thus thalidomide appears to be absorbed mainly as such, for the plasma concentrations of the drug are high initially whereas those of its hydrolysis products are low, but as time proceeds this situation is reversed.

The distribution of ¹⁴C in the embryo and maternal tissues at various times (12, 24 and 58 hr.) after the thalidomide dose is shown in Table 4. Radioactivity is present in all the tissues examined, and that in the embryo is, at nearly all the times quoted, higher than that in the plasma, brain, skeletal muscle and fat but lower than that in the liver and kidney. The distribution of ¹⁴C in the 10-day rabbit conceptus at 4 hr. after dosing the

Table 4. Distribution of ¹⁴C in the embryo and tissues of pregnant rabbits receiving [¹⁴C]thalidomide orally

Dosage was as in Table 1. The animals were killed at the times shown. N.D., Not determined.

Rabbit no.	Time (hr.)	Radioactivity (disintegrations/min./g. wet wt. of tissue)						
		Embryo	Plasma	Liver	Kidney	Brain	Muscle	Fat
7	12	1724	1421	2921	8737	N.D.	1333	747
8	12	1453	982	2079	6873	N.D.	884	542
3	24	2096	899	1490	3388	405	756	264
9	24	1517	574	917	2565	435	675	374
5	58	493	249	344	399	< 50	< 50	< 50
6	58	333	258	452	465	< 50	192	< 50

Table 5. *Distribution of radioactivity in the 10-day rabbit embryo 4 hr. after the administration of [¹⁴C]thalidomide orally to the mother*

[¹⁴C]Thalidomide (150 mg./kg.; 5 μ C/kg.) was administered orally, suspended in water, to rabbits at the 240th hour of pregnancy; conceptuses were removed from the mother 4 hr. after dosing.

Tissues examined	Radioactivity (disintegrations/min./g. wet wt. of tissue)
Complete conceptus	560* (482-667)
Embryo and membranes	658† (502-715)
Yolk-sac fluid	538† (478-625)

* Mean value of five conceptuses.

† Value obtained from pools of four conceptuses.

Table 6. *Accumulation of radioactivity in the rabbit embryo after the administration of [¹⁴C]thalidomide to the mother*

Dosage was as in Table 1; animals were killed at 4, 12, 24 or 58 hr. after dosing. Average values are given for seven to 14 embryos with the ranges in parentheses.

Time after dosing (hr.)	¹⁴ C in embryo* ¹⁴ C in maternal plasma†
4	0.54 (0.35-0.73)
12	1.36 (1.13-1.60)
24	2.41 (2.05-3.75)
58	1.57 (1.23-2.09)

* Disintegrations/min./g.

† Disintegrations/min./ml.

mother orally with thalidomide is shown in Table 5. The radioactivity is approximately uniformly distributed between the embryo and the yolk-sac fluid. Radioactivity tends to accumulate in the embryo for at least 24 hr., since the embryo ¹⁴C-maternal-plasma ¹⁴C concentration ratio increases during the first 24 hr. after dosing (see Table 6). At 4 hr. this ratio is about 0.5, increasing to 1.4 at 12 hr. and 2.4 at 24 hr.; at 58 hr. the ratio has declined to about 1.6.

Thalidomide itself is present in the embryo throughout the 24 hr. after dosing the mother, in amounts declining with time (Table 7). The primary hydrolysis products are detectable in the embryo after 4 hr. and they increase in amount, especially α -(o-carboxybenzamido)glutarimide. The secondary hydrolysis products are also detectable at 12 hr. At 24 hr. the amounts of the hydrolysis products in the embryo are slightly less than they were at 12 hr., except for 2- and 4-phthalimidoglutaramic acids, which are now slightly more. The ratios of the concentrations of thalidomide and its

Table 7. *Radioactive compounds in the rabbit embryo at various times after the administration of [¹⁴C]thalidomide to the mother*

Nine pregnant rabbits were each given [¹⁴C]thalidomide (150 mg./kg.; 5 μ C/kg.) orally on the 192nd hour of pregnancy and were killed, in threes, at 4, 12 and 24 hr. after dosing. Results are mean values for three animals. —, Not detected.

Compound determined	Concn. in embryo (μ g./g.)		
	At 4 hr.	At 12 hr.	At 24 hr.
Thalidomide	4.2	2.7	1.2
α -(o-Carboxybenzamido)- glutarimide	0.6	9.6	8.7
2- and 4-Phthalimidoglutaramic acids	0.2	1.1	1.9
2-Phthalimidoglutamic acid	< 0.1	< 0.1	< 0.1
2- and 4-(o-Carboxybenzamido)- glutaramic acids + 2-(o-carboxy- benzamido)glutaric acid	—	13.2	7.0

Table 8. *Distribution of thalidomide and its hydrolysis products between the embryo and the maternal plasma at various times after the administration of [¹⁴C]thalidomide to the mother*

—, Not detected.

Compound determined	Concn. in embryo (μ g./g.) Concn. in plasma (μ g./ml.)		
	At 4 hr.	At 12 hr.	At 24 hr.
Thalidomide	0.5	0.6	0.9
α -(o-Carboxybenzamido)glutari- mide	0.3	0.8	2.0
2- and 4-Phthalimidoglutaramic acids	—	0.5	2.3
2- and 4-(o-Carboxybenzamido)- glutaramic acids + 2-(o-carboxy- benzamido)glutaric acid	—	4.8	4.4

hydrolysis products in the embryo to those in the maternal plasma are shown in Table 8. These ratios increase with time, indicating the accumulation of thalidomide and its breakdown products in the embryo.

It is thus clear that, when thalidomide is administered orally to the mother, it can penetrate into the embryo and remains there partly as such for at least 24 hr., despite the fact that it is undergoing spontaneous hydrolysis. A possible reason for this accumulation is that, whereas thalidomide is lipid-soluble and less polar than its hydrolysis products, the latter are all polar compounds and probably less lipid-soluble at physiological pH

values. Thus thalidomide could penetrate the embryo easily and then hydrolyse spontaneously to polar products that cannot readily leave the embryo. It is thus possible that the teratogenic effect of thalidomide is mediated through one or more of its hydrolysis products, since they occur in the embryo. However, none of these products has been found to be teratogenic in the rabbit when given by mouth (Fabro, Schumacher, Stagg, Smith & Williams, 1965; Smith, Fabro, Schumacher & Williams, 1965; Keberle *et al.* 1965a; Keberle, Loustalot, Maller, Faigle & Schmid, 1965b), but this could be explained by suggesting that as such they do not penetrate into the embryo and could only be effective teratogenic agents if they are formed from thalidomide in the embryo itself. The problem of whether some of these hydrolysis products can penetrate into the embryo when given by mouth is dealt with in the succeeding paper (Fabro, Smith & Williams, 1967).

This work was supported by a grant from the Association for the Aid of Crippled Children, New York. The Packard scintillation spectrometer used in this work was purchased by means of a U.S. Public Health Service Research grant AM06772-01 from the National Institutes of Health.

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