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The Fate of the Hydrolysis Products of Thalidomide in the Pregnant Rabbit

By S. FABRO, R. L. SMITH and R. T. WILLIAMS
Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2,

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1. The fate in the pregnant New Zealand White rabbit of oral doses of four ^{14}C-labelled hydrolysis products of thalidomide, namely α-(*o*-carboxybenzamido)-glutarimide, 2-phthalimidoglutaramic acid, 2-phthalimidoglutaric acid and 2-(*o*-carboxybenzamido)glutaramic acid, administered on the 192nd hour of pregnancy has been studied. 2. About 60–95% of the administered ^{14}C of each compound appears in the urine in 58hr. and the remainder is found in the faeces and in the gut and its contents. 3. Radioactivity is present in the plasma, liver, kidney, brain, muscle, fat and embryo. 4. The ^{14}C-labelled substances in the plasma and embryo consist of the unchanged compounds and their further hydrolysis products. 5. Since the above four thalidomide hydrolysis products are found in the rabbit conceptus together with their further hydrolysis products after their oral administration to the pregnant rabbit, it appears that the teratogenic activity of thalidomide is due to the compound itself rather than to one or more of its hydrolysis products.

In the preceding paper (Fabro, Smith & Williams, 1967) it was shown that, after the oral administration of thalidomide to rabbits at the 192nd hour of pregnancy, the drug itself and seven of its hydrolysis products are to be found in the embryo. These products were found to accumulate in the embryo and to persist for more than 58hr. after dosing. The teratogenic effects of thalidomide could be due therefore to the drug itself or to one or more of these products. However, the hydrolysis products have been found to be non-teratogenic in rabbits (Fabro, Schumacher, Smith, Stagg & Williams, 1965; Smith, Fabro, Schumacher & Williams, 1965; Keberle *et al.* 1965*a*; Keberle, Loustalot, Maller, Faigle & Schmid, 1965*b*), but this lack of teratogenicity could be due to an inability to penetrate into the embryo on oral administration, for these products are all fairly polar compounds. The fate of oral doses of four of these compounds labelled with ^{14}C has been investigated in pregnant rabbits when given at the sensitive period. The compounds were 2-phthalimidoglutaramic acid, α-(*o*-carboxybenzamido)glutarimide, 2-phthalimidoglutaric acid and 2-(*o*-carboxybenzamido)glutaramic acid. It is shown below that these compounds do penetrate into the embryo when given by mouth to the mother.

MATERIALS AND METHODS

The non-radioactive hydrolysis products used have been described by Schumacher, Smith & Williams (1965).

Radioactive compounds

(±)-α-([^{14}C]o-*Carboxybenzamido)glutarimide.* α-Benzyloxycarboxamidoglutarimide (2g.) (Sondheimer & Holley, 1957) was suspended in dry methanol (50ml.) and hydrogenated for 2hr. at atmospheric pressure in the presence of palladium black (500mg.). After filtration the solution was evaporated to dryness at 40° in a rotary evaporator. The residue of α-aminoglutarimide dissolved in dry pyridine (10ml.) was treated with [*carbonyl*-^{14}C]phthalic anhydride (1·13g.; 400μc) and the mixture stirred for 3hr. After standing overnight, the pyridine was removed under reduced pressure and the residue acidified with conc. HCl. On keeping at 0° for 3hr. pink crystals of (±)-α-([*carbonyl*-^{14}C]-*o*-carboxybenzamido)glutarimide separated and were filtered off, washed with water and dried. The yield was 1·1g. (55%) with specific activity 0·2μc/mg. and m.p. 268–270° after shrinking at 200°.

(±)-2-[^{14}C]-*Phthalimidoglutaramic acid.* [*carbonyl*-^{14}C]-Phthalic anhydride (1g.; 300μc) was refluxed for 2hr. with (±)-glutamic acid (1g.) in dry pyridine (20ml.). The pyridine was removed under reduced pressure and the residue refluxed for 3min. with acetic anhydride (10ml.). The solution was evaporated under reduced pressure to a gum, which was dissolved with dry ether (60ml.) and kept at 0° overnight. Crystals of (±)-2[^{14}C]-phthalimidoglutaric anhydride separated (1·33g.). This was dissolved in warm dioxan (5ml.), and the solution cooled and treated with an excess of a saturated solution of ammonia in dry ether. The ammonium salt of (±)-2-[^{14}C]-phthalimidoglutaramic acid separated and was filtered and washed with dry ether. The salt was dissolved in water (5ml.) and the solution was made slightly acid with conc. HCl and kept overnight at 0°. (±)-2[*carbonyl*-^{14}C]-Phthalimidoglutaramic acid separated as white crystals, which were filtered off, washed with a little

cold water and dried. The yield was 1·2g. and it had m.p. 184–186° and specific activity 0·23 μc/mg.

(±)-2[^{14}C]-*Phthalimidoglutaric acid.* (±)-2(*carbonyl*-^{14}C]-Phthalimidoglutaric anhydride (1g.; 300 μc) was prepared as above and dissolved in the minimum quantity of boiling water. On cooling, crystals of (±)-2[*carbonyl*-^{14}C]-phthalimidoglutaric acid separated and were filtered off, washed with water and dried. The yield was 1·13g. with m.p. 188–189° and specific activity 0·16 μc/mg.

(±)-2-([^{14}C]o-*Carboxybenzamido)glutaramic acid* (see Schumacher, *et al.* 1965). (±)-2[^{14}C]-Phthalimidoglutaramic acid (542mg.; 125 μc) was dissolved in N-NaOH (20ml.) and the solution kept at room temperature for 15min. The solution was then treated with 20ml. of N-HCl. Portions of this solution, which contained (±)-2-([^{14}C]o-carboxybenzamido)glutaramic acid only (shown chromatographically), were administered to pregnant rabbits.

Animals and procedures

Adult New Zealand White does (body wt. 3–4kg.) as described in the preceding paper (Fabro *et al.* 1967) were used. Each compound, except (±)-2-([^{14}C]o-carboxybenzamido)glutaramic acid, which was already in solution (see above), was given orally, suspended in water (20ml.), at a dose of 150mg./kg. (5 μc/kg.) to rabbits at the 192nd hour of pregnancy and appropriate groups of animals were killed at 12, 24 and 58hr. after dosing. The collection of excreta, the sampling of tissues and embryos, the measurement of ^{14}C and the identification of compounds were carried out as described in the preceding paper (Fabro *et al.* 1967).

RESULTS

α-([^{14}C]o-*Carboxybenzamido)glutarimide.* Table 1 shows that about 80% of the ^{14}C from an oral dose of this compound is excreted in the urine during the sensitive period of 58hr., and a further 10% occurs in the faeces and the gut and its contents. The 24hr. urines collected from rabbits 13 and 14 were further examined and it was found that about 33% of the dose of ^{14}C occurs as unchanged α-(*o*-carboxybenzamido)glutarimide, about 26% as 2- plus 4-(*o*-carboxybenzamido)glutaramic acids and 3% as 2-(*o*-carboxybenzamido)glutaric acid. The plasma ^{14}C concentration reaches a maximum at 4hr. after dosing and then declines, and at 58hr. it is about 25% of the maximum (Fig. 1). The plasma radioactivity is higher at 12, 24 and 58hr. than that of other tissues, including the embryo, except the kidney (Table 2). The embryo ^{14}C/plasma ^{14}C concentration ratio is about 0·6 at 12, 24 and 58hr. after dosing. The maternal plasma and embryo contain at 24hr. after dosing α-(*o*-carboxybenzamido)glutarimide (3·9 μg./ml. and 1·9 μg./g. respectively), 2- and 4(*o*-carboxybenzamido)glutaramic acids (1·4 μg./ml. and 0·9 μg./g. respectively), 2-(*o*-carboxybenzamido)glutaric acid (0·2 μg./ml. and 0·2 μg./g. respectively) and phthalic acid (0·1 μg./ml. and 0·1 μg./g. respectively). These values are the means of three experiments.

2-[^{14}C]*Phthalimidoglutaramic acid.* At 12hr. after the oral administration of this compound to pregnant rabbits about 50% of the ^{14}C is found in the urine and 40% in the gut and its contents (Table 3). At 24 and 58hr., however, the urinary excretion of radioactivity is variable (57–84% of the dose). Similarly, the amounts found in the faeces (1·7–9·5%) and in the gut and its contents (1·0–29·8%) are variable. In the 24hr. urine, 48% of the dose is found as the unchanged compound, 5·9% as 2-phthalimidoglutaric acid, 3·6% as 2-(*o*-carboxybenzamido)glutaramic acid, 3·7% as 2-(*o*-carboxy-

Table 1. ^{14}C *in the excreta and gut of pregnant rabbits receiving* α-([^{14}C]o-*carboxybenzamido)glutarimide orally*

α-([^{14}C]o-Carboxybenzamido)glutarimide (150mg./kg.; 5 μc/kg.) was administered orally to rabbits on the 192nd hour of pregnancy.

Rabbit no.	Time (hr.)	^{14}C found (% of dose) Urine	Faeces	Gut and contents	Total
11	12	75·2	1·1	22·1	98·4
12	12	64·9	0·6	24·3	89·8
13	24	74·3*	1·1	16·4	91·8
14	24	60·1*	1·9	25·9	87·9
15	58	78·3	1·7	11·8	91·8
16	58	83·4	4·8	5·3	93·5

* These urines contained the following radioactive compounds (as % of dose; mean of values found for rabbits nos. 13 and 14): α-(*o*-carboxybenzamido)glutarimide (33·4); 2- plus 4-(*o*-carboxybenzamido)glutaramic acids (25·5); 2-(*o*-carboxybenzamido)glutaric acid (3·1).

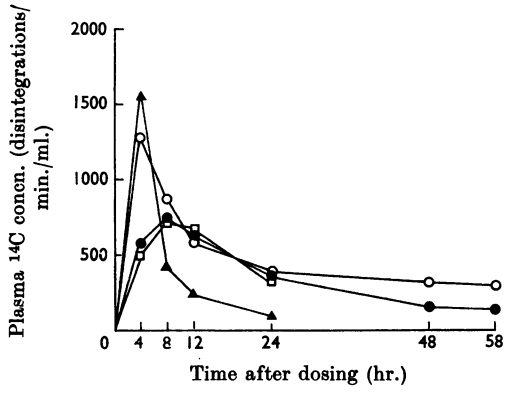


Fig. 1. Plasma ^{14}C concentrations of pregnant New Zealand White rabbits after the oral administration (150mg./kg.; 5 μc/kg.) of α-([^{14}C]o-carboxybenzamido)glutarimide (○), 2[^{14}C]-phthalimidoglutaramic acid (●), 2[^{14}C]-phthalimidoglutaric acid (▲) and 2-([^{14}C]o-carboxybenzamido)glutaramic acid (□) on the 192nd hour of pregnancy.

Table 2. ^{14}C *in the tissue and embryos of pregnant rabbits receiving* α-([^{14}C]-o-*carboxybenzamido)glutarimide orally*

Dosage and animals were as in Table 1. The animals were killed at the times indicated. Each value for tissues is the mean of duplicates, and for embryo the mean of three or more.

		Radioactivity (disintegrations/min./g. wet. wt. of tissue)						
Rabbit no.	Time (hr.)	Embryo	Plasma	Liver	Kidney	Brain	Muscle	Fat
11	12	562	859	846	8468	<15	105	55
12	12	330	566	163	3179	<15	73	<50
13	24	316*	469	209	2674	<15	75	230
14	24	267*	446	259	1780	<15	86	302
15	58	225	338	320	2438	<15	70	50
16	58	152	276	234	1867	<15	<50	<15

* The specific activity of the embryo with its membranes and of the yolk-sac fluid were determined in these cases and found to be the same.

Table 3. ^{14}C *in the excreta and gut of pregnant rabbits receiving* 2[^{14}C]-*phthalimidoglutaramic acid orally*

2[^{14}C]-Phthalimidoglutaramic acid (150 mg./kg.; 5 μc/kg.) was administered orally to pregnant rabbits on the 192nd hour of pregnancy.

		^{14}C found (% of dose)			
Rabbit no.	Time (hr.)	Urine	Faeces	Gut and contents	Total
31	12	49·8	0·1	39·9	89·8
32	12	55·9	0·4	38·3	94·6
34	24	67·9*	7·4	16·6	91·9
35	24	57·3*	1·7	29·8	88·8
36	58	62·0	8·4	25·0	95·4
37	58	83·8	9·5	1·0	94·3

* These urines contained the following radioactive compounds (as % of dose; mean of values found for rabbits nos. 34 and 35): 2-phthalimidoglutaramic acid (48); 2-phthalimidoglutaric acid (5·9); 2-(o-carboxybenzamido)glutaramic acid (3·6); 2-(o-carboxybenzamido)glutaric acid (3·7); phthalic acid (0·4).

benzamido)glutaric acid and 0·4% as phthalic acid. The plasma ^{14}C concentration reaches a maximum at about 8hr. after dosing (Fig. 1). At 12, 24 and 58hr. after dosing the plasma ^{14}C concentration is higher than that found in other tissues except the kidney (Table 4). The embryo ^{14}C/plasma ^{14}C concentration ratio is in the range 0·35–0·075. The maternal plasma and embryo contain at 24hr. after dosing 2-phthalimidoglutaramic acid (5·8 μg./ml. and 2·5 μg./g. respectively), 2-phthalimidoglutaric acid (0·1 μg./ml. and 0·1 μg./g. respectively), 2-(o-carboxybenzamido)glutaramic acid (0·7 μg./ml. and 0·4 μg./g. respectively) and 2-(o-carboxybenzamido)glutaric acid (0·4 μg./ml. and 0·1 μg./g. respectively). These values are the means of three experiments.

2[^{14}C]-*Phthalimidoglutaric acid.* When this acid is administered orally to pregnant rabbits about 90% of the ^{14}C is excreted in the urine in 24hr. and about 5% is found in the faeces and in the gut and its contents. About a half of the dose of this acid is excreted unchanged in the urine in 24hr. but 23% of the dose appears as 2-(o-carboxybenzamido)-glutaric acid (Table 5). The plasma ^{14}C concentration is highest at 4hr. after dosing and then rapidly declines, and at 24hr. only traces of ^{14}C are found in the plasma (Fig. 1). The maternal plasma and embryo contain at 8hr. after dosing 2-phthalimidoglutaric acid (4·7 μg./ml. and 2·8 μg./g. respectively), 2-(o-carboxybenzamido)glutaric acid (0·4 μg./ml. and 0·3 μg./g. respectively) and phthalic acid (<0·1 μg./ml. and <0·1 μg./g. respectively). These values are the means of three experiments.

2-([^{14}C]o-*Carboxybenzamido)glutaramic acid.* After the oral administration of this compound to rabbits at the 192nd hour of pregnancy, about 58% of the ^{14}C appears in the urine in 24hr. and 34% in the faeces and gut and its contents. Analysis of the 24hr. urine shows that about 47% of the dose is excreted unchanged, together with small amounts of 2-(o-carboxybenzamido)glutaric acid (2·4%) and phthalic acid (7·9%) (Table 6). The plasma ^{14}C concentration is highest at about 8hr. after dosing and at 24hr. it has dropped to about half the maximum value (Fig. 1). Table 7 shows the tissue distribution of ^{14}C at 24hr. after an oral dose of the glutaramic acid. The plasma ^{14}C concentration is greater than that of the embryo and maternal tissues except the kidney. The maternal plasma and embryo contain at 24hr. after dosing 2-(o-carboxybenzamido)glutaramic acid (4·0 μg./ml. and

Table 4. ^{14}C *in the tissues and embryos of pregnant rabbits receiving 2[^{14}C]-phthalimidoglutaramic acid orally*

Dosage and animals were as in Table 3. The animals were killed at the times indicated and the radioactivity of the tissues was determined. Each value for tissues is the mean of two determinations, and for embryo the mean for three or more. N.D., Not determined, —, not detected.

		Radioactivity (disintegration/min./g. wet wt. of tissue)						
Rabbit no.	Time (hr.)	Embryo	Plasma	Liver	Kidney	Brain	Muscle	Fat
31	12	219	498	336	11476	<50	127	N.D.
32	12	185	329	111	3424	<50	80	77
34	24	272	361	148	3955	122	59	62
35	24	270	832	429	6620	<50	60	62
36	58	79	246	66	1770	—	<50	—
37	58	129	<50	—	413	—	70	84

Table 5. *Excretion of ^{14}C by pregnant rabbits after receiving 2[^{14}C]-phthalimidoglutaric acid orally*

2[^{14}C]-Phthalimidoglutaric acid (150mg./kg.; 5μc/kg.) was administered orally to pregnant rabbits on the 192nd hour of pregnancy. The composition of the urinary ^{14}C was as follows (% of dose): 2-phthalimidoglutaric acid (55·3); 2-(*o*-carboxybenzamido)glutaric acid (23·2); phthalic acid (2·7). These are mean values for the three animals.

	^{14}C found at 24hr. after dosing (% of dose)			
Rabbit no.	Urine	Faeces	Gut and contents	Total
52	92·0	0·5	5·2	97·7
53	81·2	1·6	7·8	90·6
54	94·4	0·2	2·7	97·3

Table 6. *Excretion of ^{14}C by pregnant rabbits receiving 2-([^{14}C]o-carboxybenzamido)glutaramic acid orally*

2-([^{14}C]o-Carboxybenzamido)glutaramic acid (150mg./kg.; 5μc/kg.) was administered orally on the 192nd hour of pregnancy. The 24hr. urine contained the following radioactive compounds (as % of dose; mean value for the two animals): 2(*o*-carboxybenzamido)glutaramic acid (46·7); 2-(*o*-carboxybenzamido)glutaric acid (2·4); phthalic acid (7·9).

	^{14}C found at 24hr. after dosing (% of dose)			
Rabbit no.	Urine	Faeces	Gut and contents	Total
73	60·4	13·8	19·4	93·6
74	55·7	17·2	18·1	91·0

3·6μg./g. respectively), 2-(*o*-carboxybenzamido)-glutaric acid (<0·1μg./ml. and <0·1μg./g. respectively) and phthalic acid (0·5μg./ml. and 0·3μg./g. respectively). These values are the means of three experiments.

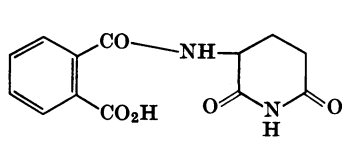
DISCUSSION

In the preceding paper (Fabro *et al.* 1967) it was shown that, if thalidomide is given orally to pregnant rabbits at the time when the embryo is sensitive to the teratogen, the drug itself and seven of its hydrolysis products are present in the conceptus at 12 and at 24hr. after dosing. In fact, at 12hr. after treatment the concentration of the hydrolysis products in the embryo is about nine times that of thalidomide. These findings raise the question of the significance of the presence in the embryo of these hydrolysis products in the teratogenic action of thalidomide. The hydrolysis products of thalidomide do not appear to be teratogenic in the pregnant rabbit when given by mouth (Fabro *et al.* 1965; Smith *et al.* 1965; Keberle *et al.* 1965*a*,*b*), but these findings are not conclusive since it is not known whether the hydrolysis products, when given orally, can penetrate into the embryo. Indeed, little is known about the transmission of foreign compounds from the mother to the embryo during morphogenesis. The present work shows that, of the seven hydrolysis products of thalidomide that have been found in the rabbit embryo after the oral administration of the drug to the mother, four, namely α-(*o*-carboxybenzamido)glutarimide (I), 2-phthalimidoglutaramic acid (II), 2-phthalimidoglutaric acid (III) and 2-(*o*-carboxybenzamido)glutaramic acid (IV), can penetrate into the conceptus when given to the mother by mouth. Further, two other hydrolysis products, namely 4-(*o*-carboxybenzamido)glutaramic acid and 2-(*o*-carboxybenzamido)glutaric acid, have been found in the rabbit embryo after some of these products have been given. Thus 4-(*o*-carboxybenzamido)glutaramic acid is found in the embryo after the administration of α-(*o*-carboxybenzamido)glutarimide, and 2-(*o*-carboxybenzamido)glutaric acid is present in the embryo after the administration of 2-phthalimidoglutaramic acid, α-(*o*-carboxybenzamido)glutar-

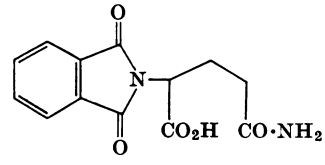
Table 7. *Radioactivity in the tissues and embryo of pregnant rabbits 24 hr. after receiving 2-([^{14}C]o-carboxybenzamido)glutaramic acid orally*

Dosage was as in Table 6. Each value for tissues is the mean of duplicate estimations, and for embryo the mean for three or more. —, Not detected.

	Radioactivity (disintegrations/min./g. wet wt. of tissue)						
Rabbit no.	Embryo	Plasma	Liver	Kidney	Brain	Muscle	Fat
73	408	410	50	4517	—	67	50
74	264	315	—	1302	—	242	172



(I)



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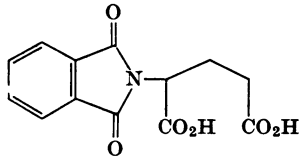

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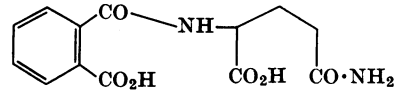

(IV)

imide, 2-phthalimidoglutaric acid or 2-(*o*-carboxybenzamido)glutaramic acid to the mother.

The ability of the remaining hydrolysis product, namely 4-phthalimidoglutaramic acid, to penetrate into the embryo has not been studied, but it is probable that it can penetrate since the chemically related 2-phthalimidoglutaramic acid and 2-phthalimidoglutaric acid are able to do so when administered orally.

Since it has been shown that six of the hydrolysis products found in the embryo of the rabbit after the oral administration of thalidomide to the mother can themselves penetrate into the embryo, and since none of these products are teratogenic when given orally to the mother, it appears improbable that the teratogenic activity of thalidomide is due to one or more of these breakdown products. This conclusion leads to the suggestion that thalidomide itself is the teratogenic agent.

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