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THE METABOLISM OF THALIDOMIDE: THE FATE OF THALIDOMIDE AND SOME OF ITS HYDROLYSIS PRODUCTS IN VARIOUS SPECIES

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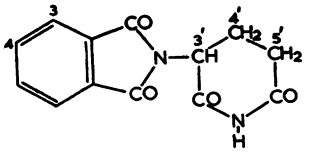
When thalidomide is administered orally to animals, only a small amount of the unchanged drug is excreted in the urine (for example, Beckman, 1962). The major portion of the compound is broken down and excreted as transformation products. After administration of the drug to man, rats and rabbits, Smith, Williams & Williams (1962) isolated 4-phthalimidoglutaramic acid from rabbit urine and detected it in human and rat urine. They also noted the presence in the urine of a fluorescent metabolite which they considered to be related to 3-hydroxyphthalic acid. Using [^{14}C]-thalidomide, Faigle, Keberle, Riess & Schmid (1962) showed that the urine of dogs given thalidomide contained 2- and 4-phthalimidoglutaramic acid, 2-phthalimidoglutaric acid, α-(*o*-carboxybenzamido)glutarimide, 2-(*o*-carboxybenzamido)glutaric acid, phthalic acid and small amounts of unchanged thalidomide, whilst the faeces contained only unchanged thalidomide.

In order to emphasize the complexity of the problem of identifying the metabolites of thalidomide it should be pointed out that there are twelve possible hydrolysis products of (±)-thalidomide of which eleven can occur in (+)- and (−)-forms. The number of possible optically active products, including (+)- and (−)-thalidomide, is thus twenty-four. If thalidomide is hydroxylated *in vivo*, this could occur in the 3- or 4-position of the aromatic ring and possibly in three different positions (3′, 4′ and 5′) in the glutarimide ring (see formula). Disregarding hydroxylation of the glutarimide ring and considering only the hydroxylation of the aromatic ring, there are twelve 3-hydroxy- and twelve 4-hydroxy-metabolites possible, and twenty-two of these can occur in (+)- and (−)-forms, the two compounds not containing an optically active carbon atom being 3- and 4-hydroxy-



phthalic acids. The number of possible metabolites of thalidomide is thus very large, for if any racemic metabolite underwent resolution the possible number of metabolites would be well over 100. In the present paper we describe the isolation and detection of some fifteen urinary metabolites and prove that rabbit urine contains derivatives of 3- and 4-hydroxyphthalic acid.

METHODS

Reference compounds

5-Hydroxyphthalazine-1,4-dione. 3-Hydroxyphthalimide was converted into 5-hydroxyphthalazine-1,4-dione by refluxing with aqueous hydrazine according to Drew & Pearman (1937). The hydroxy-compound was recrystallized from a large volume of hot water to give almost white rods, melting point (m.p.) 328 to 330° C (decomposition). Drew & Pearman (1937) give m.p. 330° C (decomp.). In ethanol solution, the compound gave a reddish-brown colour with ferric chloride solution and fluoresced a bright blue when viewed in ultraviolet light.

6-Hydroxyphthalazine-1,4-dione. 4-Nitrophthalimide was prepared by nitration of phthalimide (Blatt, 1943) and converted into 4-aminophthalimide, m.p. 294° C (decomp.), according to Levy & Stephen (1931). The amino-compound was then converted into the 4-hydroxyphthalimide, m.p. 288 to 290° C, by diazotization and boiling as described by Drew & Pearman (1937) for the preparation of the 3-hydroxy-isomer. The 4-hydroxy-compound was converted by hydrazinolysis to 6-hydroxyphthalazine-1,4-dione, which was obtained from hot water as white crystals, m.p. 390 to 393° C. Curtius & Hoesch (1907) give the m.p. as being above 300° C. In ethanol solution the compound showed a pale blue fluorescence in ultraviolet light.

Phthalazine-1,4-dione, m.p. 342 to 344° C, was purchased (Light & Co., Colnbrook, Bucks). It gave a pale blue fluorescence in solution when illuminated with ultraviolet light. The above three phthalazine-diones were readily separated by chromatography using the two-dimensional method described in the preceding paper (Schumacher, Smith & Williams, 1965). The chromatographic behaviour of these compounds is shown in Table 1.

TABLE 1

CHROMATOGRAPHIC SEPARATION OF PHTHALAZINE-1,4-DIONE AND ITS HYDROXY-DERIVATIVES

The chromatograms (Whatman No. 1), using about 10 μg of each compound in acetone, were first developed with solvent A, and then at right angles in solvent B (see Schumacher *et al.*, 1965); R_F values are in the two dimensions. Fluorescence was in ultraviolet light

Compound	R_F values in A then B	Fluorescence
Phthalazine-1,4-dione	0·61, 0·68	Pale blue
5-Hydroxyphthalazine-1,4-dione	0·49, 0·73	Bright blue
6-Hydroxyphthalazine-1,4-dione	0·64, 0·61	Pale blue

2-(3-Hydroxyphthalimido)glutaric acid. 3-Hydroxyphthalic acid (3 g; m.p. 146 to 148° C) was refluxed for 2 hr with 50 ml. of toluene in a flask fitted with a Dean and Stark trap to remove water. Evaporation of the toluene under reduced pressure left 3 g of crude 3-hydroxyphthalic anhydride. The latter (2 g) was mixed with DL-glutamic acid (2 g) and heated under reflux (Dean & Stark trap) with 100 ml. of toluene for 2 hr. The toluene was evaporated and the residue was recrystallized from water. Four successive fractions of crystals were obtained: (1) 1.7 g, m.p. 160 to 165° C, (2) 0.42 g, m.p. 209 to 211° C, (3) 0.15 g, m.p. 146 to 153° C, and (4) 0.15 g, m.p. 180 to 181° C. On chromatography on paper, only fraction (2) contained the required material as indicated by a green fluorescence in ultraviolet light. Fraction (2) was recrystallized from water until it was free from 3-hydroxyphthalic acid, which shows a blue fluorescence in ultraviolet light. Eventually, 0.33 g of 2-(3-hydroxyphthalimido)glutaric acid was obtained which showed one spot on chromatography and a green fluorescence. It had m.p. 215 to 216° C. (Found: C, 52.7; H, 4.0; N, 5.2%. $C_{13}H_{11}O_7N$ requires C, 53.2; H, 3.8; N, 4.8%.)

Isolation and detection of urinary metabolites in rabbits

By solvent extraction

Thalidomide (1.5 g) was administered orally suspended in water to each of three rabbits (3 to 4 kg body wt.). These animals refused food and water for the following two days, but did not exhibit signs of central nervous depression. Little urine was passed during this time, but eventually a dark reddish-brown concentrated urine (total 180 ml., *p*H 7 to 8 by indicator paper) was passed and collected in flasks containing a little toluene.

α-Aminoglutarimide. The urine was shaken with ether (200 ml., twice) and the reddish extract dried over anhydrous sodium sulphate. On evaporation, the extract yielded a small amount (8 mg) of pale reddish crystals. These appeared to be α-aminoglutarimide, for they showed identical R_F values (0.44 and 0.13, respectively, in solvents A and B) with authentic material and gave a characteristic pink colour with ninhydrin.

α-(o-Carboxybenzamido)glutarimide. The residual urine was clarified with charcoal (2 g), filtered and evaporated to a small volume in a rotary evaporator at 45° C and at reduced pressure. The concentrated urine was then extracted with methanol (200 ml., twice). The extracts were filtered, evaporated almost to dryness, and the residue was treated with acetone (200 ml.). A crystalline fraction separated which on chromatography was shown to contain four compounds, namely α-(*o*-carboxybenzamido)glutarimide, 2- and 4-(*o*-carboxybenzamido)glutaramic acids and 2-(*o*-carboxybenzamido)glutaric acid. The crystals were collected and dissolved in methanol (50 ml.) and the solution was evaporated to 20 ml. and water (60 ml.) was added. Crystals separated and these were recrystallized from hot aqueous methanol to give white crystals of α-(*o*-carboxybenzamido)glutarimide (300 mg), m.p. 273° C after losing water at 200° C. The crystals, which were soluble in bicarbonate solution with effervescence, showed R_F 0.37 and 0.63 in solvents A and B respectively, identical with the authentic compound. (Found: C, 55.7; H, 4.5; N, 10.7; O, 30.7%. $C_{13}H_{12}N_2O_5$ requires: C, 56.5; H, 4.3; N, 10.1; O, 29.0%.) On incubating the compound at *p*H 7.4 and 37° C for 4 hr and chromatographing the resultant solution, phthalic acid, α-aminoglutarimide, glutamine and isoglutamine were detected.

The methanol mother liquor from the above compound was shown to contain 2- and 4-(*o*-carboxybenzamido)glutaramic acid and 2-(*o*-carboxybenzamido)glutaric acid by paper chromatography in the solvents A and B.

Phthalic acid. The acetone filtrate, after removal of the *o*-carboxybenzamido compounds mentioned above, was chromatographed and found to contain 2- and 4-phthalimidoglutaramic acids, 2-phthalimidoglutaric acid and phthalic acid. It was shaken with charcoal (1 g), filtered and the filtrate concentrated to a few ml. The concentrated material was dissolved in water (50 ml.) and on standing phthalic acid separated. It was recrystallized from aqueous methanol to give white crystals (68 mg), m.p. and mixed m.p. 218 to 219° C. (Found: C, 57.9; H, 3.6; O, 38.7%. $C_8H_6O_4$ requires: C, 57.8; H, 3.6; O, 38.5%.)

2-Phthalimidoglutaric acid. The aqueous filtrate from the phthalic acid was concentrated under reduced pressure to 10 ml. and then shaken with ethyl acetate (25 ml.) and warmed. The aqueous phase was separated and allowed to stand at 0° C overnight. Crystals of 2-phthalimidoglutaric acid (82 mg) separated and were recrystallized from hot water. The white crystals had m.p. 198° C and mixed m.p. 198 to 199° C. The infrared spectrum and R_F value in solvent A were identical with those of 2-phthalimidoglutaric acid. (Found: C, 55.8; H, 4.0; N, 5.1; O, 34.7%. $C_{13}H_{11}NO_6$ requires: C, 56.3; H, 4.0; N, 5.1; O, 34.6%.)

2-Phthalimidoglutaramic acid. The ethyl acetate phase (20 ml.), described in the preceding paragraph, was diluted with more ethyl acetate (10 ml.) and kept overnight at 0° C. Crystals of 2-phthalimidoglutaramic acid (34 mg) separated and were recrystallized from a mixture of methanol and ethyl acetate. They had m.p. and mixed m.p. 188 to 189° C, and the infrared spectrum was identical with that of the authentic compound. (Found: C, 55.8; H, 4.3; N, 9.1; O, 29.5%. $C_{13}H_{12}N_2O_5$ requires: C, 56.5; H, 4.3; N, 10.1; O, 29.0%.)

4-Phthalimidoglutaramic acid. The ethyl acetate mother liquor described in the preceding paragraph was concentrated to 5 ml. and light petroleum (boiling point 40 to 60° C) was added until the solution was just turbid. On keeping at 0° C overnight, 4-phthalimidoglutaramic acid (148 mg) crystallized out. The crystals were filtered and recrystallized from aqueous methanol to give white crystals, m.p. 178 to 179° C and mixed m.p. 178° C. The infrared spectrum was identical with that of authentic material. (Found: C, 56.6; H, 4.5; N, 9.7; O, 30.1%. $C_{13}H_{12}N_2O_5$ requires: C, 56.5; H, 4.3; N, 10.1; O, 29.0%.)

By adsorption chromatography

Thalidomide (0.45 g/kg) was fed to three rabbits (3 to 4 kg body weight). The animals refused food and water and passed no urine for 24 to 36 hr. The 48-hr urine (730 ml. including cage washings) was dark brown in colour with *p*H about 6.5 and was filtered through glass-wool. It was then evaporated to a syrup at 45 to 50° C and reduced pressure in a rotary evaporator. An alumina column (50 g of grade H activated

Alumina 100/200s mesh; Peter Spence, Widnes) was prepared using a mixture of benzene and chloroform (3 : 1) as solvent. The syrup (25 g) was placed at the top of the column which was then developed with the benzene-chloroform mixture to which increasing amounts of an equal mixture by volume of dioxan, ethyl methyl ketone, acetone, methanol and chloroform were added. Initially 1% of the latter solvent mixture was used and then the amounts were increased in order to 2.5, 5, 10, 20 and finally 100%. Twenty fractions (10 to 200 ml.) were collected and each one was examined for fluorescence and quenching under ultraviolet light. Each fraction was evaporated at 40° C and reduced pressure and the concentrated material was allowed to stand at room temperature. Three fractions, Nos. 3, 10 and 12, were found to contain materials related to thalidomide.

Fraction 3, on standing, deposited crystals of thalidomide (42 mg), m.p. and mixed m.p. 271° C. The identity was checked by infrared spectroscopy. (Found: C, 60.5; H, 3.9; N, 10.8; O, 24.7%. $C_{13}H_{10}N_2O_4$ requires: C, 60.5; H, 3.9; N, 10.85; and O, 24.8%.) Fraction 10 was an oil which on chromatography was shown to contain 2- and 4-phthalimidoglutaramic acids and 2-phthalimidoglutaric acid. These were separated by thin-layer chromatography on alumina in solvent A. Ten such thin-layer plates (8 × 8 in.) were used to isolate these compounds. The area of alumina on each plate containing the appropriate compound was scraped off the plate and eluted with warm methanol. The eluates were filtered, concentrated and set aside to crystallize. There was isolated in this way 4-phthalimidoglutaramic acid (40 mg), m.p. and mixed m.p. 178 to 179° C, 2-phthalimidoglutaramic acid (10 mg), m.p. 188 to 189° C and mixed m.p. 188° C, and 2-phthalimidoglutaric acid (12 mg), m.p. and mixed m.p. 198 to 199° C.

Fraction 12 was also an oil and was found to contain the amino acids. These were also separated by thin-layer chromatography on alumina using solvent A. Appropriate areas corresponding to the amino acids from 20 chromatograms were scraped off and the alumina was eluted with aqueous methanol (10 ml.). The eluates were filtered and allowed to stand at room temperature whereby each fraction crystallized. The crystals in each case were recrystallized from aqueous methanol and there was obtained DL-glutamic acid (20 mg), m.p. and mixed m.p. 225° C (decomp.), whose identity was checked by infrared spectroscopy (found: C, 40.7; H, 6.2; N, 9.5; O, 43.8%. $C_5H_9NO_4$ requires: C, 40.8; H, 6.2; N, 9.5; O, 43.5%), DL-glutamine (20 mg), m.p. and mixed m.p. 230° C (decomp.) and the identity checked by infrared spectroscopy (found: C, 41.0; H, 6.8; N, 19.2; O, 32.75%. $C_5H_{10}O_3N_2$ requires: C, 41.0; H, 6.9; N, 19.2; O, 32.8%), and DL-isoglutamine (18 mg), m.p. and mixed m.p. 188 to 192° C (decomp.), which gave a violet colour with ninhydrin and its infrared spectrum and chromatographic mobility in solvents A and B were identical with that of a synthetic sample of DL-isoglutamine.

RESULTS

Examination of faeces in rabbits

The faeces of the three rabbits, which had received 0.45 g/kg of thalidomide orally as described in Methods, were collected for 4 days. The bulked faeces were then extracted with dioxan (250 ml.) in a Soxhlet apparatus under reduced pressure at 60° C. The extract was filtered and evaporated to dryness on a rotary evaporator at 45° C and reduced pressure. The residue was extracted with ether (3 × 100 ml.) followed by water (150 ml.) to remove pigments and then dissolved in dioxan (100 ml.).

The dioxan solution was concentrated at 45° C under reduced pressure to a small volume which was diluted with water and allowed to stand. Thalidomide separated and was recrystallized from 2-methoxyethanol to give white crystals (112 mg), m.p. and mixed m.p. 271° C. Its identity was checked by infrared spectrum. (Found: C, 60.3; H, 3.85; N, 10.7; O, 25.25%. $C_{13}H_{10}O_4N_2$ requires C, 60.5; H, 3.9; N, 10.85; O, 25.8%.)

A similar sample of faeces in another experiment was extracted with ether (400 ml.) in a Soxhlet apparatus for 3 hr. The extract was filtered and concentrated. The concentrated solution was placed on an alumina column (50 g grade H as before) prepared with benzene-methanol and the column developed with the same solvent containing

increasing amounts of methanol. Seven fractions were collected and nos. 5 to 7 were combined and taken almost to dryness at 45° C under reduced pressure. The residue was treated with water and allowed to stand. Crystals (120 mg) separated and were identified as α-(*o*-carboxybenzamido)glutarimide. They were recrystallized from aqueous methanol and on heating they lost water at 200° C and melted at 273° C. When mixed with authentic α-(*o*-carboxybenzamido)glutarimide, the m.p. behaviour was the same. The infrared spectrum and R_F values of the compound in solvents A and B were identical with those of the authentic compound.

Metabolism of some thalidomide hydrolysis products in rabbits

4-*Phthalimidoglutaramic acid.* This compound (1 g) was fed to each of two rabbits (3.6 and 3.9 kg body weight). The 12-hr urines (*p*H 5.6 and 5.8) were collected (total 50 ml.). No urine was passed for the next 24 hr, but another total of 200 ml. (*p*H 5.8) was obtained after 48 hr. Both urines (12- and 48-hr) contained crystalline deposits of unchanged 4-phthalimidoglutaramic acid. Chromatography showed that the urines contained also 4-(*o*-carboxybenzamido)glutaramic acid, phthalic acid and isoglutamine.

The bulked urines were first filtered through coarse glass wool and then through filter paper to remove from the deposit unchanged 4-phthalimidoglutaramic acid which, after recrystallization from aqueous methanol, had m.p. and mixed m.p. 178 to 179° C.

The urine was now evaporated nearly to dryness at 45° C under reduced pressure and the residue extracted with methanol (2×200 ml.) which was then treated with charcoal, filtered and evaporated to dryness. The residue was extracted with acetone (200 ml.) to give an acetone-extract and a residue (see below) consisting of 4-(*o*-carboxybenzamido)-glutaramic acid, urea, inorganic salts and small amounts of amino acids. The acetone extract was evaporated almost to dryness and water was added whereby phthalic acid separated (m.p. and mixed m.p. 218° C after recrystallization from aqueous methanol) in a yield of 60 mg (5% of dose). The aqueous filtrate from the phthalic acid, on keeping at 0° C overnight, yielded 600 mg (30% of dose) of 4-phthalimidoglutaramic acid, m.p. 178° C, after recrystallization. (Found: C, 56.5; H, 4.5; N, 10.0; O, 30.7%. Calculated for $C_{13}H_{12}N_2O_5$: C, 56.5; H, 4.3; N, 10.1; O, 29.0%.)

The residue from the acetone-extract (see above) which was shown chromatographically to contain 4-(*o*-carboxybenzamido)glutaramic acid was dissolved in water (30 ml.), and the solution was treated with 2 N-sodium hydroxide (3 ml.) and methanol (60 ml.). On keeping the solution overnight at 0° C, the disodium salt of 4-(*o*-carboxybenzamido)-glutaramic acid separated. It was purified by repeated solution in water and precipitation with methanol (yield 120 mg or 5% of dose).

2-*Phthalimidoglutaramic acid.* This compound (1 g) was fed to a rabbit (3.9 kg) and a 24-hr urine specimen (70 ml., *p*H 6) collected. On chromatography, the urine was shown to contain the original compound and 2-(*o*-carboxybenzamido)glutaramic acid and phthalic acid. After filtering through glass wool, the urine was evaporated nearly to dryness at 45° C under reduced pressure and the residue was extracted with acetone (200 ml.) and 2-(*o*-carboxybenzamido)glutaramic acid separated (49 mg or 4.9% of dose) and was purified as the disodium salt as in the case of its isomer above.

The acetone-filtrate was taken to dryness and the residue was treated with water, and, on keeping at 0° C, phthalic acid (m.p. and mixed m.p. 219° C) separated (28 mg or 4.7% of dose) and, on further standing, 2-phthalimidoglutaramic acid separated (180 mg or 18% of the dose), m.p. 184 to 186° C.

2-*Phthalimidoglutaric acid.* This acid (1 g) was fed to a rabbit (4.4 kg) and the 24-hr urine (80 ml., *p*H 6.1) was collected. Chromatography showed it to contain the compound fed, 2-(*o*-carboxybenzamido)glutaric acid and phthalic acid. These were isolated by the same extraction procedures and yielded 2-phthalimidoglutaric acid, m.p. and mixed m.p. 198 to 199° C in a yield of 212 mg (21% of dose), phthalic acid (90 mg or 15% of dose) and 2-(*o*-carboxybenzamido)glutaric acid (120 mg or 12% of dose). The last compound was isolated as its trisodium salt which was purified by solution in water and precipitation with methanol.

4-(o-*Carboxybenzamido)glutaramic acid.* A total of 2.75 g of this compound was fed to six rabbits (dose, 50 to 250 mg/kg). Two animals died in 2.5 hr and a third 3 days after dosing. The other animals were sluggish in behaviour. The urine from four animals after 12 hr (total volume 320 ml., *p*H 8 to 8.3) contained thick precipitates of inorganic phosphates. On chromatography, the urine contained the original compound, phthalic acid and isoglutamine. From the urine after adjusting the *p*H to 6.5 with 2 N-hydrochloric acid, there was isolated by extraction of the evaporated urine with acetone the compound fed (purified as the disodium salt) and phthalic acid (10% of dose).

2-(o-*Carboxybenzamido)glutaramic acid.* The disodium salt of this compound (1 g) was fed to a rabbit (4.2 kg) and the 24-hr urine sample was collected (82 ml., *p*H 7.6). The urine was acidified to *p*H 6 with 2 N-hydrochloric acid, evaporated as before, and the residue was extracted with methanol. There was isolated 186 mg (18.6% of dose) of the unchanged compound and 72 mg (13.5% of dose) of phthalic acid. Paper chromatography also revealed the presence of the unchanged compound and phthalic acid.

2-(o-*Carboxybenzamido)glutaric acid.* The trisodium salt of this acid (1 g) was fed to a rabbit (3.3 kg) and the 24-hr urine sample (58 ml., *p*H 6.2) was collected. By a similar procedure as in the other experiments, namely evaporation to dryness and methanol extraction, there was isolated the unchanged acid as the trisodium salt (220 mg or 22% of dose) and phthalic acid (28 mg or 5.8% of dose).

α-*Aminoglutarimide.* The metabolism of this compound has been investigated in rats. Three female rats (180 to 200 g body weight) each received α-aminoglutarimide hydrochloride (500 mg in 2 ml. of water) by intraperitoneal injection. Urine samples (12 and 24 hr) were collected in flasks containing a few drops of concentrated hydrochloric acid and 5 ml. of toluene. In the absence of the hydrochloric acid the voided urine was dark bluish-green in colour and this changed to a deep ink-blue colour on standing. The urine of the α-aminoglutarimide-fed rats regularly stained the skin and tails of the animals a deep blue colour.

Paper chromatography of urine samples (0.03 ml.) using the two-dimensional system revealed the presence of at least six ninhydrin positive spots. One of these was unchanged α-aminoglutarimide and three of the other five spots were identified as glutamine, isoglutamine and glutamic acid. A seventh spot was deep blue in colour and ninhydrin negative.

The 24-hr urine sample (20 ml.) from the above rats was evaporated to dryness using a rotary evaporator and the residue was dried overnight in a vacuum desiccator. The powder was applied to a Celite column (10 g) and the column developed with benzene-methanol (3 : 1). The methanol concentration was gradually increased until finally pure methanol was used. Thirteen fractions (10 ml.) were collected and examined chromatographically.

From fraction 1 a blue compound was isolated (12 mg). This substance was insoluble in water but soluble in benzene, methanol and ether. Its structure is unknown but it gave no reaction with ninhydrin indicating loss of the α-amino function. Fraction 2 was shown chromatographically to contain glutamine, isoglutamine and glutamic acid together with inorganic material.

Chromatographic studies

The chromatographic investigations described below were carried out using Whatman No. 1 paper and the solvent systems A and B described in the preceding paper. These solvents were used separately and in combination to give two-dimensional chromatograms, solvent A being used first followed by drying of the paper which was then irrigated with solvent B in a direction at right angles to that of A.

Chromatography of urine and faeces of rabbits given thalidomide

Urine. Urine samples from rabbits were clarified by filtration first through glass wool and then through filter paper. Aliquots of 0.1 to 0.5 ml. of the filtered urine were used for chromatography with the two-dimensional system. The findings are shown in Table 2.

TABLE 2

CHROMATOGRAPHY OF URINE FROM RABBITS GIVEN THALIDOMIDE

Procedure is described in the text. R_F values are × 100 of spots found in the two-dimensional system (see text), A first and then B. * In ultraviolet light

No.	R_F × 100	Compound identified	Remarks
I	75–88, 60–70	Thalidomide	
II	37–43, 55–59	4-Phthalimidoglutaramic acid	Detected as described in (Schumacher *et al.*, 1965)
III	27–34, 48–55	2-Phthalimidoglutaramic acid	
IV	10–39, 39–42	α-(*o*-Carboxybenzamido)glutarimide	
V	22–26, 65–75	2-Phthalimidoglutaric acid	
VI	9–25, 22–27	4-(*o*-Carboxybenzamido)glutaramic acid	
VII	12–26, 32–37	2-(*o*-Carboxybenzamido)glutaramic acid	
VIII	0– 3, 12–25	2-(*o*-Carboxybenzamido)glutaric acid	
IX	55–58, 55–60	Phthalic acid	
X	0–40, 7–12	α-Aminoglutarimide	
	7–20, 60–63	Possibly 2-(3-hydroxyphthalimido)glutaric acid	Green fluorescence*
	8–16, 55–60	Unknown	Bluish-green fluorescence*
	18–21, 50–53	Unknown	Yellowish-blue fluorescence*
	70–73, 66–70	Unknown	Quenching spot*

Faeces. Two rabbits (4.3 and 4.4 kg body weight) were each given 1 g of thalidomide orally and the faeces were collected for the following 7 days. The faeces (520 g) were extracted with methanol (1 l.) by homogenizing in a large Waring blender. The extract was filtered and concentrated to a small bulk (25 ml.). At this stage some greenish pig-

ments separated and were removed by filtration. The filtrate was concentrated to 5 ml., and aliquots of this were used for paper chromatography. A similar extract was prepared from the faeces of rabbits which had not received thalidomide and was used as a control in interpreting the chromatograms. The results are shown in Table 3.

TABLE 3

CHROMATOGRAPHY OF FAECES FROM RABBITS GIVEN THALIDOMIDE

Extracts of faeces were prepared as described in the text and chromatographed on Whatman No. 1 paper using solvent A first and then solvent B at right angles. The R_F values quoted are the ranges observed for each spot

$R_F \times 100$	Compound identified	Remarks
75–85, 58–64	Thalidomide	Main component of the faecal extract
35–45, 55–60	4-Phthalimidoglutaramic acid	Detected as described in the text
23–30, 65–72	2-Phthalimidoglutaramic acid	
17–35, 35–41	α-(*o*-Carboxybenzamido)glutarimide	
22–37, 43–48	2-Phthalimidoglutaric acid	
0–41, 10–12	α-Aminoglutarimide	

Chromatography of tissues and intestinal contents of rats dosed with thalidomide

Brain. Thalidomide (400 mg/kg) was fed to eight female rats (170 to 190 g). After 1.5 hr, the rats were stunned and bled into a beaker containing heparin solution (0.5 ml.). The brains were removed immediately and homogenized in 200 ml. of equal volumes of cold acetone and methanol in a Waring blender. The homogenate was centrifuged for 10 min at 2,000 revs/min, and the clear supernatant fluid was separated and evaporated to dryness at 35° C under diminished pressure in a rotary evaporator. The residue was dissolved in 10 ml. of the acetone-methanol mixture and again evaporated as before to 1 ml. This was cooled and then centrifuged to remove the protein material which had separated. An aliquot (0.05 to 0.15 ml.) of the clear supernatant fluid was used for chromatography, the results of which are shown in Table 4. The brains from six rats which had not received thalidomide were similarly processed as a control. The major component in brain appeared to be thalidomide.

Plasma. The heparinized blood (48 ml.) obtained above was centrifuged for 10 min and the plasma (about 20 ml.) carefully removed and poured into 250 ml. of the same acetone-methanol solvent. The mixture was agitated in a Waring blender and then centrifuged to remove proteins. The supernatant fluid was evaporated to dryness at 35° C as above and the residue dissolved in 10 ml. of the same solvent, and then evaporated to 1 ml. On cooling, protein separated and was removed, and the clear extract was chromatographed as above. The results are shown in Table 4.

Gastrointestinal contents. Three rats were killed 2 hr after an oral dose of thalidomide (400 mg/kg). The gastrointestinal tracts were carefully dissected and their contents removed. The bulked contents were extracted with two 200-ml. samples of equal volumes of acetone and methanol. After centrifuging, the extracts were evaporated as above and the residue was dissolved in 20 ml. of the same solvent. This was concentrated as before and an aliquot was used for two-dimensional chromatography. The results are shown in Table 5.

TABLE 4

CHROMATOGRAPHY OF RAT PLASMA AND BRAIN AFTER THALIDOMIDE

Extracts of plasma and brain were prepared as described in text, and then subjected to two-dimensional chromatography with solvent A and then B on Whatman No. 1 paper. R_F values $\times 100$ are of spots found in two dimensions, direction A first and then B

$R_F \times 100$ in		
Plasma	Brain	Compound identified
78–88, 66–73	77–86, 65–72	Thalidomide
32–42, 55–59	36–45, 58–62	4-Phthalimidoglutaramic acid
12–47, 32–37	12–43, 35–38	α-(*o*-Carboxybenzamido)glutarimide
22–29, 67–74	27–33, 69–76	2-Phthalimidoglutaramic acid
10–40, 7–12	13–40, 9–11	α-Aminoglutarimide

TABLE 5

CHROMATOGRAPHY OF RAT GASTROINTESTINAL CONTENTS 2 HR AFTER ORAL DOSING WITH THALIDOMIDE

The extract was prepared as described in the text. R_F values are $\times 100$ of spots found in two dimensions, A first and then B

$R_F \times 100$	Compound identified	Remarks
82–96, 63–73	Thalidomide	Major component
30–53, 55–62	4-Phthalimidoglutaramic acid	
31–36, 47–57	2-Phthalimidoglutaramic acid	
10–38, 44–47	α-(*o*-Carboxybenzamido)glutarimide	
12–32, 71–74	2-Phthalimidoglutaric acid	
10–25, 20–22	4-(*o*-Carboxybenzamido)glutaramic acid	
10–24, 35–38	2-(*o*-Carboxybenzamido)glutaramic acid	Identification uncertain
0–3, 10–25	2-(*o*-Carboxybenzamido)glutaric acid	
57–61, 55–60	Phthalic acid	
10–41, 10–12	α-Aminoglutarimide	

The metabolism of thalidomide in the rat, guinea-pig and mouse

The urinary metabolites of thalidomide in the rat, guinea-pig and mouse have been investigated using paper chromatography and the reactions previously described to identify the metabolic products. The effect of urinary *p*H on the nature of the metabolites has been investigated in rats.

Rats. Five groups of three rats (females; 150 to 180 g body weight) were used. Group 1 was given thalidomide orally (400 mg/kg); group 2 was given orally 100 mg of sodium acetate per rat in 0.5 ml. of water followed 30 min later by thalidomide (400 mg/kg); group 3 was given orally 100 mg of ammonium chloride per rat in 0.5 ml. of water followed 30 min later by thalidomide (400 mg/kg). Groups 4 and 5 were controls, the former receiving sodium acetate (100 mg) only and the latter ammonium chloride (100 mg) only, and the urines from these groups were used on control chromatograms. The urines from all these animals were collected for 20 hr, and a sample (0.1 ml.) from each group was chromatographed on paper using the two-dimensional system. The approximate *p*H of the urine was determined by means of indicator paper (Johnson's comparator paper, Hendon Ltd.). The results of this experiment are shown in Table 6, which shows that when the urine *p*H is below 7, α-(*o*-carboxybenzamido)glutarimide is the major metabolite found in the urine and, when the *p*H is about 8, 4-phthalimidoglutaramic acid is the major metabolite, together with 2- and 4-(*o*-carboxybenzamido)glutaramic acids.

Mice. Ten female mice (20 g) were each given 1 g/kg of thalidomide suspended in Tragacanth gum. At this dose level, two of the mice died during the course of 24 hr.

TABLE 6

EFFECT OF URINARY *p*H UPON THE NATURE OF THE MAJOR METABOLITE OF THALIDOMIDE IN RAT URINE

* See text. Numbers for compounds refer to the list in Table 2

Group	Treatment*	*p*H of urine*	Major urinary metabolite	Other compounds detected
1	Thalidomide only	6·5	α-(*o*-Carboxybenzamido)-glutarimide (No. IV)	I, II, III, V, X
2	Sodium acetate, thalidomide	7·5–8·0	4-Phthalimidoglutaramic acid (No. II)	III, IV, V, VI, VII, VIII, IX, X
3	Ammonium chloride, thalidomide	6·5–7·0	α-(*o*-Carboxybenzamido)-glutarimide (No. IV)	II, III, V, X and traces of VI and VII

The 24-hr urine (12 ml., *p*H 8.0) was collected, filtered and rapidly taken to dryness using a rotary evaporator at 40° C. The residue was extracted with methanol (15 ml.), and the extract was filtered and evaporated as before to 2 to 3 ml. Aliquots (0.1 ml.) of the extract were used for chromatography. The major hydrolysis product in the urine was 4-phthalimidoglutaramic acid. Other hydrolysis products identified were unchanged thalidomide, 2-phthalimidoglutaramic acid, 2-phthalimidoglutaric acid, phthalic acid, α-(*o*-carboxybenzamido)glutarimide and α-aminoglutarimide.

Guinea-pigs. Two guinea-pigs (female, 900 g body weight) each received 300 mg of thalidomide orally. The 24-hr urine output (37 ml., *p*H 7.2) was collected, filtered and used directly for chromatography.

The major hydrolysis product present was 4-phthalimidoglutaramic acid; also identified were α-(*o*-carboxybenzamido)glutarimide, 2-phthalimidoglutaramic acid, 2-phthalimidoglutaric acid, α-aminoglutarimide and phthalic acid.

The detection of phenolic compounds derived from thalidomide in rabbit urine

Thalidomide (1.5 g) was fed to each of two rabbits (weight 3 kg) and their urines were collected for 48 hr. The combined urines (total volume, 110 ml., both with *p*H 8) were filtered through glass wool and then concentrated at 45° C to 20 ml. using a rotary evaporator. The urine was refluxed for 2 hr with an equal volume of 20 ml. of concentrated hydrochloric acid, cooled and then extracted for 6 hr with ether in a continuous extractor. The ether-extract was removed, dried with anhydrous sodium sulphate and concentrated to about 5 ml., when crystals of phthalic acid separated. The crystals were filtered off and the mother liquor was used for two-dimensional paper chromatography using solvents A and B. An ether-extract of hydrolysed rabbit urine was similarly prepared for control purposes.

Chromatography of the thalidomide urine-extract revealed the presence of a small bright blue fluorescent band R_F 18–32/59–62 corresponding to 3-hydroxyphthalic acid and a larger light blue fluorescent spot R_F 31–43/53–60 of 4-hydroxyphthalic acid. When the paper was irrigated with a dilute solution of ferric chloride the two areas reacted to give reddish-brown spots.

In a second experiment thalidomide (1 g) was fed to each of four rabbits (3.5 to 4.5 kg) and the urine (170 ml., *p*H 7.8) was collected for 36 hr. The filtered urine was adjusted to *p*H 3 with 2 N-hydrochloric acid and then evaporated under reduced pressure almost to dryness. The residue was extracted with methanol (200 ml.) and then with 200 ml. of a

mixture of acetone and methanol (1 : 1). The mixed extracts after filtration were evaporated as before almost to dryness, and the residue was taken up in water (20 ml.) and refluxed with hydrazine hydrate (1 g of 95%) for 1 hr. During this time, some phthalazine-1,4-dione separated. The solution was cooled and the precipitate was separated by filtration. Aliquots of both the precipitate and filtrate were then chromatographed two-dimensionally using solvents A and B separately. All three chromatograms showed the presence of phthalazine-1,4-dione and of 5- and 6-hydroxyphthalazine-1,4-dione in the filtrate and precipitate.

The formation of phenolic metabolites was also investigated using [^{14}C]-thalidomide. Two rabbits (3.5 kg body weight) received 500 mg/kg of thalidomide orally containing 10 μc of [^{14}C]-thalidomide. The 48-hr urines (120 and 80 ml.) were collected separately, filtered and concentrated to 30 ml. on a rotary evaporator. The urines were refluxed with an equal volume of concentrated hydrochloric acid for 2 hr, cooled and then extracted with ether for 4 hr. The ether-extract was dried with anhydrous sodium sulphate and concentrated to a small volume (3 ml.). Aliquots were chromatographed as before and the areas containing 3- and 4-hydroxyphthalic acid were cut out and radioactivity was measured in a scintillation counter. Both areas were radioactive, the spot containing the 4-hydroxy-derivative being about four-times more active than the area carrying the 3-hydroxy-compound.

DISCUSSION

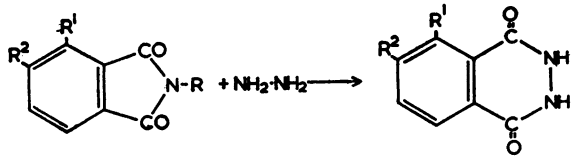
In the preceding paper (Schumacher *et al.*, 1965) it was shown that if thalidomide in solution encountered a *p*H of 6.0 or greater, then it underwent spontaneous hydrolysis. It is therefore not surprising that the spontaneous hydrolysis products of thalidomide have been found in the urine and faeces of rabbits and in the brain, blood and gastrointestinal contents of rats after oral dosing with thalidomide. In rabbit urine, all the spontaneous hydrolysis products of thalidomide shown in Fig. 1 of the preceding paper have been found chromatographically (Table 2), and, by solvent extraction and column chromatography of the urine, most of them have been isolated in crystalline form. Many of these compounds have also been found in the faeces, so that *p*H values sufficiently high to allow spontaneous hydrolysis must have been encountered. In rabbit faeces, the major product was thalidomide itself, but the three primary hydrolysis products, 2- and 4-phthalimidoglutaramic acids and α-(*o*-carboxybenzamido)glutarimide, and two secondary hydrolysis products, 2-phthalimidoglutaric acid and α-aminoglutarimide, were also detected (Table 3). In fact, we were able to isolate α-(*o*-carboxybenzamido)glutarimide from the faeces in crystalline form. On examination of the gut contents of rats which had received thalidomide orally 2 hr previously, the major component was found to be thalidomide, but we were also able to detect chromatographically nine hydrolysis products (Table 5). These findings suggest that thalidomide is, at least, partially decomposed before it is absorbed from the gastrointestinal tract, although the major compound in the gut is unchanged thalidomide.

In the brain and plasma of rats, we were able to detect thalidomide and four other products (Table 4), that is two primary and two secondary hydrolysis products. In the brain extract, thalidomide appeared to be the major compound and this is not unexpected since all the hydrolysis products of thalidomide are carboxylic acids and are polar and therefore are unlikely to cross the blood-brain barrier as readily as thalidomide, which is relatively nonpolar. It is possible that the hydrolysis products found in the brain are

formed *in situ* from thalidomide which has already penetrated into the brain. It seems likely therefore that, after an oral dose of thalidomide, there will be circulating in the blood thalidomide itself and twelve spontaneous hydrolysis products.

The extent to which thalidomide breaks down could depend upon a number of factors such as the *p*H encountered, the time after dosing, tissue binding, and the occurrence of hydrolases (in the gut or tissues) which may assist any one of the spontaneous hydrolyses. Examination of Fig. 1 in the previous paper shows there are twenty separate hydrolyses, three primary, seven secondary, seven tertiary and three quaternary according to the number of hydrolytic steps from thalidomide. Any one of these could be assisted by a hydrolase which might vary with the species of animal. At present, however, enzymic hydrolysis of thalidomide has not been investigated. Tissue binding could also be a factor in protecting thalidomide from hydrolysis but again this aspect has not been investigated.

We stated in an earlier paper (Smith *et al.*, 1962) that thalidomide may be hydroxylated *in vivo* in the aromatic ring. We have now obtained evidence which supports this view. In the first place we obtained fluorescent spots suggestive of phenolic hydroxy-compounds on chromatograms of urine from rabbits (Table 2); one of these was possibly 2-(3-hydroxyphthalimido)glutaric acid (which we had synthesized) in view of its R_F value and fluorescence properties. In the second place we found that urine extracts treated with hydrazine and then chromatographed gave fluorescent spots corresponding to 5- and 6-hydroxyphthalazine-1,4-dione, compounds of known structure. The phthalazinediones are readily formed from phthalimide derivatives, but less readily from phthalamic acids, thus:



where $R^1=R^2=H$ in thalidomide and phthalazine-1,4-dione, $R^1=OH$, $R^2=H$ in 3-hydroxythalidomide and 5-hydroxyphthalazinedione and $R^1=H$, $R^2=OH$ in 4-hydroxythalidomide and 6-hydroxyphthalazinedione. Finally, from the hydrolysed urine of rabbits dosed with [^{14}C]-thalidomide it was possible to demonstrate chromatographically the presence of small amounts of radioactive 3- and 4-hydroxyphthalic acids.

Fluorescent hydroxy-compounds were not found in the urine of rabbits dosed with some of the hydrolysis products of thalidomide, six of which were fed as described in the experimental section; they were found only after thalidomide. This finding is not unexpected, for if the hydroxylation is carried out by liver microsomes as in the case of other aromatic compounds (Brodie & Hogben, 1957; Brodie, Gillette & La Du, 1958) then only thalidomide, being lipoid soluble and relatively nonpolar, would be expected to penetrate the microsomes readily. The hydrolysis products are carboxylic acids which could be expected to be ionized at physiological *p*H values. Aromatic hydroxylation is an enzymic reaction and therefore one could expect species differences in the extent of the hydroxylation of thalidomide. It has been shown recently in this laboratory that the 7-hydroxylation of coumarin, for example, depends upon species, for it occurs in liver microsomes from man

and the rabbit but not from the rat (Creaven, Parke & Williams, 1962). The possibility that the hydroxythalidomides could be teratogenic metabolites of thalidomide is suggested by the report of Boylen, Horne & Johnson (1963, 1964) that synthetic 3- and 4-hydroxythalidomides produce abnormalities in chicks, if they are injected into the incubated egg.

It was shown in the preceding paper that the amide bonds present in the phthalimide and glutarimide rings of thalidomide exhibit slightly different *p*H sensitivities as regards hydrolysis. Above *p*H 7 both rings are hydrolysed while below this *p*H only the phthalimide amide bonds suffer hydrolysis. The results with rats treated with sodium acetate or ammonium chloride before administration of thalidomide indicate that the *p*H of the urine may influence the nature of the hydrolysis products present. Thus normal rats and rats treated with ammonium chloride excreted mainly α-(*o*-carboxybenzamido)glutarimide while sodium acetate treatment resulted in the excretion of larger amounts of 4-phthalimidoglutaramic acid. It is difficult to interpret these findings in the light of the known hydrolysis pattern of thalidomide. Even at *p*H 8 when significant amounts of 4-phthalimidoglutaramic acid are formed the major route of hydrolysis is still by cleavage of the phthalimide ring. However, these results suggest that species differences in respect of the amounts of the various hydrolysis products excreted may be at least partly related to differences in *p*H environment encountered by the drug *in vivo*.

SUMMARY

1. The metabolic fate of thalidomide has been investigated in the rabbit, rat, mouse and guinea-pig.

2. The metabolites of thalidomide present in the urine, blood and tissues of various species dosed with the drug were characterized by comparing their chromatographic mobility and colour reactions with those given by authentic samples of the compounds. In the case of the rabbit, the urinary metabolites were isolated in crystalline form by means of solvent extraction and adsorption chromatography, and their identity was established by analysis, melting point behaviour and the comparison of their infrared spectra with those of the authentic compounds.

3. When thalidomide is fed to rabbits, rats, mice and guinea-pigs a number of hydrolysis products appear in the urine. These hydrolysis products are those formed by the spontaneous hydrolysis of thalidomide described in the preceding paper. In addition the urine of rabbits dosed with thalidomide contains derivatives of 3- and 4-hydroxyphthalic acid; these are minor metabolites and have not been identified. The hydrolysis products appear to be derived by spontaneous breakdown of thalidomide in the body, although it is possible that any of the hydrolytic reactions of thalidomide may be assisted by hydrolases in the body.

4. In rats, some breakdown of thalidomide occurs in the gut before absorption, for hydrolysis products are present in the gastrointestinal tract following the oral administration of the drug.

5. Both thalidomide and some of its hydrolysis products can be detected in the plasma and brain of rats dosed orally with thalidomide.

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REFERENCES

BECKMANN, R. (1962). Über das Verhalten von Thalidomid im Organismus. *Arzneimittel-Forsch.*, **12**, 1095–1098.

BLATT, A. H. (1943). *Organic Syntheses*, Coll. Vol. 2, p. 459. New York: J. Wiley & Son.

BOYLEN, J. B., HORNE, H. H. & JOHNSON, W. J. (1963). Teratogenic effects of thalidomide and related substances. *Lancet*, i, 552.

BOYLEN, J. B., HORNE, H. H. & JOHNSON, W. J. (1964). Teratogenic effects of thalidomide and its metabolites on the developing chick embryo. *Canad. J. Biochem.*, **42**, 35–42.

BRODIE, B. B., GILLETTE, J. R. & LA DU, B. N. (1958). Enzymatic metabolism of drugs and other foreign compounds. *Ann. Rev. Biochem.*, **27**, 427–454.

BRODIE, B. B. & HOGBEN, C. A. M. (1957). Some physico-chemical factors in drug action. *J. Pharm. Pharmacol.*, **9**, 345–380.

CREAVEN, P. J., PARKE, D. V. & WILLIAMS, R. T. (1962). Aromatic hydroxylation by liver microsomes. *Biochem. J.*, **85**, 5–6*P*.

CURTIUS, T. & HOESCH, A. (1907). Die Einwirkung von Hydrazinehydrat auf Nitroverbindungen. *J. prakt. Chem.*, **76**, 301–330.

DREW, H. D. K. & PEARMAN, F. H. (1937). Chemiluminescent organic compounds, part II. The effect of substituents on the closure of phthalhydrazides to 5- and 6-membered rings. *J. chem. Soc.*, 26–33.

FAIGLE, J. W., KEBERLE, H., RIESS, W. & SCHMID, K. (1962). The metabolic fate of thalidomide. *Experientia (Basel)*, **18**, 389–397.

LEVY, L. F. & STEPHEN, H. (1931). The preparation of 4-nitrophthalimide and derivatives. *J. chem. Soc.*, 79–82.

SCHUMACHER, H., SMITH, R. L. & WILLIAMS, R. T. (1965). The metabolism of thalidomide. The spontaneous hydrolysis of thalidomide in solution. *Brit. J. Pharmacol.*, **25**, 324–337.

SMITH, R. L., WILLIAMS, R. A. D. & WILLIAMS, R. T. (1962). Phthaloylisoglutamine, a metabolite of thalidomide. *Life Sciences*, **7**, 333–336.