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THE METABOLISM OF THALIDOMIDE: THE SPONTANEOUS HYDROLYSIS OF THALIDOMIDE IN SOLUTION

BY

H. SCHUMACHER, R. L. SMITH AND R. T. WILLIAMS

From the Department of Biochemistry, St. Mary's Hospital Medical School, London, W.2

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The sedative, neurotoxic and embryotoxic effects of (±)-α-phthalimidoglutarimide or thalidomide are now well known. Any of these effects could be due to thalidomide, or to its metabolites, and it is therefore important to identify these metabolites and to study their biological properties. Our studies on thalidomide began with an investigation of the urinary metabolites excreted by rabbits dosed orally with thalidomide and, as will be described in the succeeding paper (Schumacher, Smith & Williams, 1965), it was soon found that a large number of metabolites occurred. In fact, we succeeded in isolating or detecting by colour reactions and paper chromatography all twelve of its possible hydrolysis products.

This caused us to suspect that thalidomide might be unstable in solution and in this paper we shall describe the conditions for the spontaneous hydrolysis of thalidomide in aqueous solution at various *p*H values.

In describing the spontaneous hydrolysis and metabolites of thalidomide, it will be useful to refer to Fig. 1, which shows how thalidomide could break down by simple hydrolysis of its substituted amide bonds.

METHODS AND RESULTS

Reference compounds

These compounds were either obtained as gifts or prepared in this laboratory. A list is given in Table 1 of the sources of reference compounds, their melting points and any other relevant data. The following compounds were synthesized and their properties are described.

2- *and* 4-(o-*carboxybenzamido)glutaramic acid and* 2-(o-*carboxybenzamido)glutaric acid and their sodium salts.* (±)-4-Phthalimidoglutaramic acid (5 g) was stirred into an amount of aqueous sodium hydroxide (40% w/v) calculated to yield the disodium salt of (±)-4-(*o*-carboxybenzamido)glutaramic acid. The greenish solution was allowed to stand at room temperature for 10 min and then methanol (100 ml.) was added. The mixture was kept for 4 to 5 hr. The precipitate which had formed was filtered, washed with methanol and then recrystallized five times from aqueous methanol and dried in a desiccator. The disodium salt of (±)-4-(*o*-carboxybenzamido)glutaramic acid was obtained as a white crystalline powder in a yield of 80 to 82%. (Found: C, 46.1; H, 3.5; N, 8.2; O, 28.0;

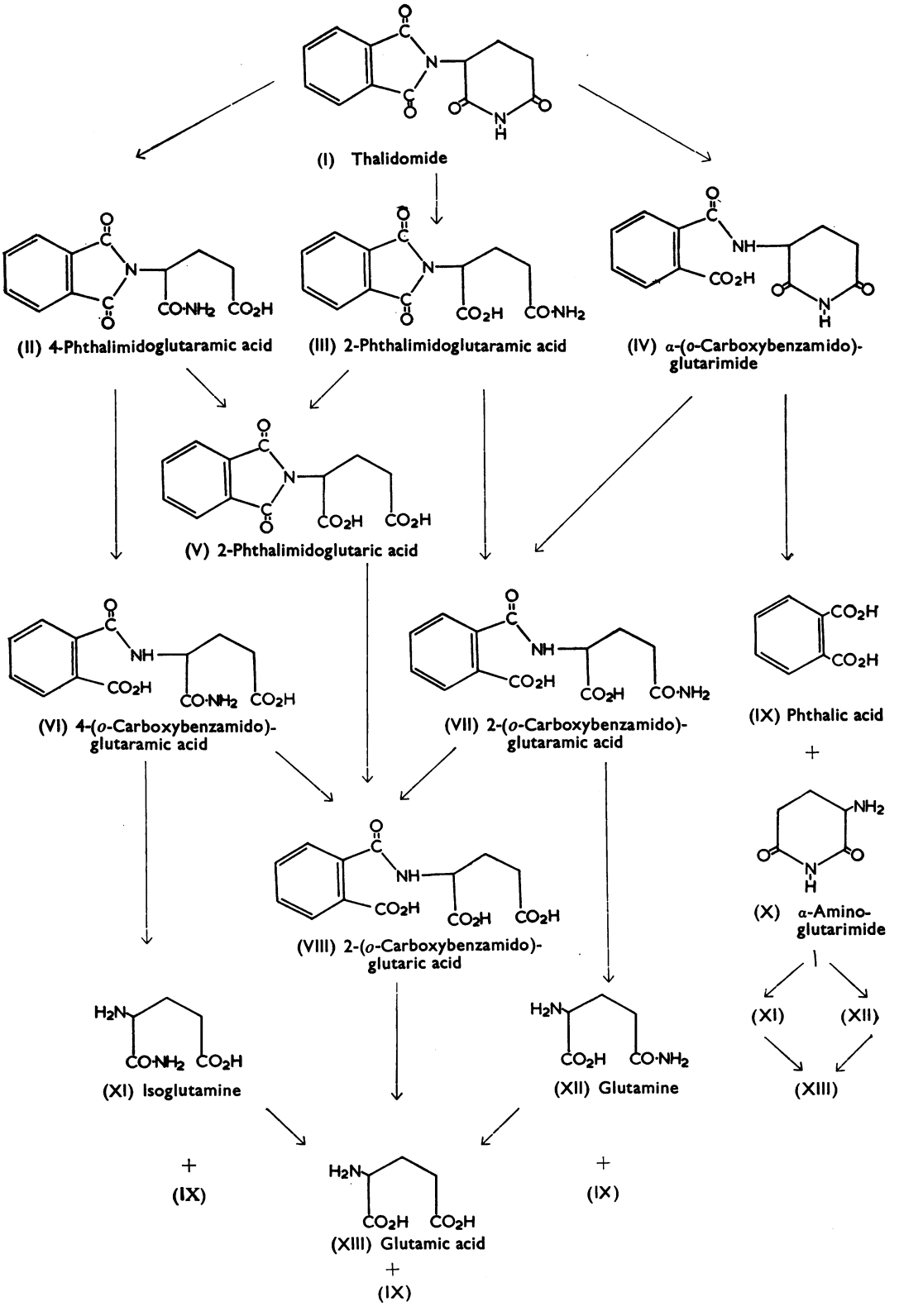

Fig. 1. The spontaneous hydrolysis of thalidomide.

TABLE 1

REFERENCE COMPOUNDS RELATED TO THALIDOMIDE

D = Gift of Distillers Co. Ltd; C = gift of Ciba Ltd., Basel; S = synthesized in this laboratory; P = purchased, B.D.H. Ltd; m.p.= melting point; * decomposition

No.	Compound	Source	Melting point (°C)	Remarks and references
I	(±)-Thalidomide	D	271	—
Ia	(−)-Thalidomide	D	256–258	$[\alpha]_D^{20}$ −45·7° in dimethylformamide
Ib	(+)-Thalidomide	D	275–277	$[\alpha]_D^{20}$ +53·2° in dimethylformamide
II	(±)-4-Phthalimidoglutaramic acid	D	178–179	King, Jackson & Kidd (1951) give m.p. 170–172° C
III	(±)-2-Phthalimidoglutaramic acid	D	184–186	King & Kidd (1949) give m.p. 194–195° C
IV	(±)-α-(*o*-Carboxybenzamido)glutarimide	C	273	This compound loses water at 200° C and cyclizes to thalidomide, and the m.p. recorded is that of thalidomide
V	(±)-2-Phthalimidoglutaric acid	S	198–199	Prepared according to King & Kidd (1949), who give m.p. 189–190° C
VI	(±)-4-(*o*-Carboxybenzamido)glutaramic acid	S	167–168*	Prepared by hydrolysis of compound II
VII	(±)-2-(*o*-Carboxybenzamido)glutaramic acid	S	Softens at 114–116, melts at 154*	Prepared by hydrolysis of compound III
VIII	(±)-2-(*o*-Carboxybenzamido)glutaric acid	S	168–170*	Prepared by hydrolysis of compound V
IX	Phthalic acid	P	219	—
X	(±)-α-Aminoglutarimide hydrochloride	C, S	—	Sondheimer & Holley (1957)
XI	DL-Isoglutamine	S	188–192	Prepared from compound II according to King *et al.* (1951), who say it chars above 180° C
XII	DL-Glutamine	P	250–256*	—
XIII	DL-Glutamic acid	P	223–227*	—

Na, 13.65%. $C_{13}H_{12}N_2O_6Na_2$ requires C, 46.2; H, 3.5; N, 8.3; O, 28.4; Na 13.6%.) The salt (1 g) was dissolved in a minimum of cold water and the solution was acidified to *p*H 2 with concentrated hydrochloric acid. The free acid was precipitated with acetone (20 ml.) and the whole was kept at 0° C overnight. The precipitated acid was purified by dissolving in the minimum of water and precipitating with an equal mixture of acetone and methanol and repeating the process five times. The yield of (±)-4-(*o*-carboxybenzamido)glutaramic acid was 42% and it had a melting point (m.p.) 167 to 168° C (decomp.). (Found: C, 53.2; H, 4.7; N, 9.5; O, 31.8%. $C_{13}H_{14}N_2O_6$ requires C, 53.1; H, 4.8; N, 9.5; O, 32.6%.)

The above procedure was repeated with (±)-2-phthalimidoglutaramic acid and there was obtained in similar yield the disodium salt of (±)-2-(*o*-carboxybenzamido)glutaramic acid (found: C, 46.0; H, 3.5; N, 8.3; O, 27.95; Na, 13.7%. $C_{13}H_{12}N_2O_6Na_2$ requires C, 46.2; H, 3.5; N, 8.3; O, 28.4; Na, 13.6%) and the free acid, m.p. 154° C (decomp.) after softening at 114 to 116° C. (Found: C, 53.3; H, 4.7; N, 9.65; O, 32.0%. $C_{13}H_{14}N_2O_6$ requires C, 53.1; H, 4.8; N, 9.5; O, 32.6%.)

The procedure was again repeated with (±)-2-phthalimidoglutaric acid and there was obtained in similar yields the trisodium salt of (±)-2-(*o*-carboxybenzamido)glutaric acid. (Found: C, 43.3; H, 2.75; N, 3.9; O, 31.0; Na, 19.0%. $C_{13}H_{10}O_7NNa_3$ requires C, 43.2; H, 2.8; N, 3.9; O, 31.0; Na, 19.1%.) From this salt the free acid was prepared with m.p. 168 to 170° C (decomp.). (Found: C, 52.7; H, 4.5; N, 4.7; O, 37.7%. $C_{13}H_{13}NO_7$ requires C, 54.1; H, 4.5; N, 4.85; O, 38.8%.)

These compounds were shown by paper chromatography to be single compounds free from the parent phthalimido derivatives and from the amino acids, isoglutamine, glutamine or glutamic acid.

Spectrophotometric study of spontaneous hydrolysis

Thalidomide in aqueous ethanol or dilute acid exhibits two absorption peaks in the ultraviolet region of the spectrum. An intense absorption occurs at 220 mμ and a weak one at about 300 mμ (Beckmann & Kampf, 1961; Green & Benson, 1961). At an alkaline *p*H the phthalimide ring opens and this is accompanied by a marked fall in the extinction at 220 mμ. Thus the hydrolytic cleavage of this ring can be followed by estimating the decrease in optical density at 220 mμ of an aqueous solution of thalidomide.

Preparation of solution. A saturated aqueous solution of thalidomide was prepared by shaking about 50 mg of the compound with 50 ml. of distilled water for 3 min and filtering. The final concentration of this solution (estimated spectrophotometrically) was 45 to 50 μg/ml., taking the $E^{1\%}_{1cm}$ at 220 mμ as 1,950 (Green & Benson, 1961).

Conditions for quantitative hydrolysis of the phthalimide ring. The phthalimide ring of thalidomide was completely hydrolysed after exposure to an excess of N-sodium hydroxide for 10 min. Volumes of an aqueous solution of thalidomide containing 73 to 294 μg of the compound were treated with a large excess (5 ml.) of N-sodium hydroxide for 10, 20, 30 and 60 min. The solutions were neutralized with N-hydrochloric acid (5 ml.), diluted to 50 ml. with water and the optical density at 225 mμ (see below) was measured. Identical aliquots were treated with a mixture of 5 ml. of N-sodium hydroxide with 5 ml. of N-hydrochloric acid, diluted to 50 ml. with water and the extinction at 225 mμ was observed to give

the effect of zero time of hydrolysis. A 10-min exposure to the alkali produced a maximal fall in extinction.

Reference curve for the hydrolysis of thalidomide. A saturated aqueous solution of thalidomide was prepared and the concentration of drug estimated spectrophotometrically (Unicam SP 500 Spectrophotometer) at 220 mμ. Aliquots (1 to 4 ml.) of the solution were treated for 15 min with N-sodium hydroxide (5 ml.) followed by N-hydrochloric acid (5 ml.) then diluted to 50 ml. with 0.2 M-tris [2-amino-2-(hydroxymethyl)propane-1,3-diol hydrochloride] buffer, *p*H 7.4, and the extinction at 225 mμ was measured. This gave a series of optical densities termed E_1. An identical series of aliquots were treated with 5 ml. of N-sodium hydroxide neutralized with 5 ml. of N-hydrochloric acid diluted to 50 ml. as before, and the optical densities were recorded to give a second series of values termed E_2. A graph was plotted relating optical density change on hydrolysis ($E_2 - E_1$) and concentration of thalidomide. The optical densities were measured at 225 mμ and not at the absorption peak of thalidomide (220 mμ) because this allowed more favourable instrumental settings of the spectrophotometer.

Effect of p*H on the spontaneous hydrolysis.* The effect of *p*H on the rate of hydrolysis of thalidomide at 37° C was studied. A saturated aqueous solution of the drug was prepared and the concentration determined spectrophotometrically. A series of dilutions

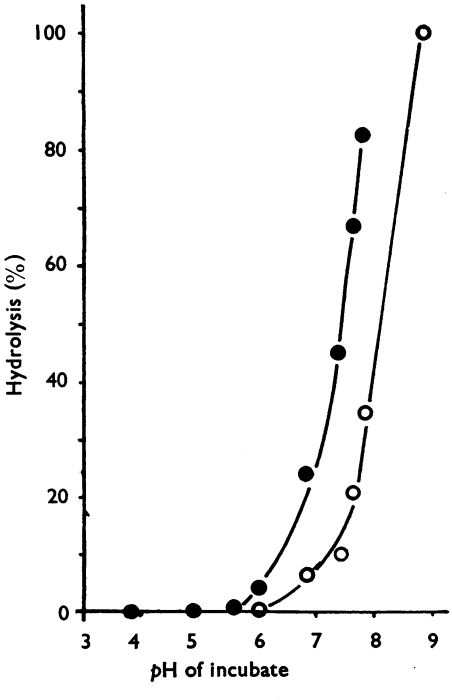

Fig. 2. Effect of *p*H on the hydrolysis of thalidomide. Ordinate: hydrolysis, %; abscissa: *p*H of incubate. ○——○ 1 hr incubation; ●——● 5 hr incubation.

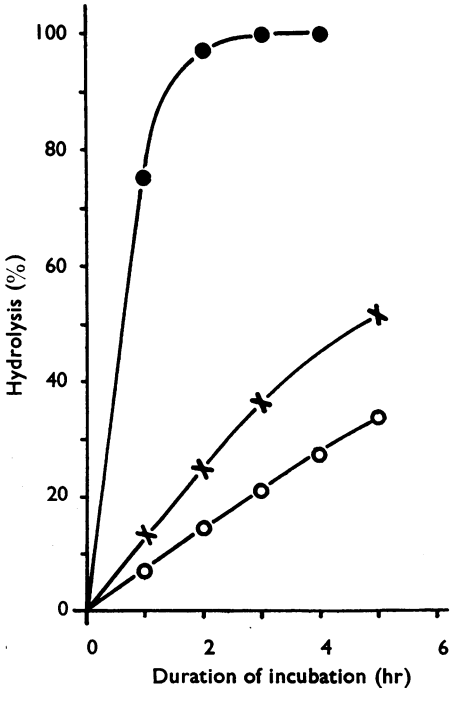

Fig. 3. Rate of hydrolysis of thalidomide at *p*H 7, 7.4 and 8. Ordinate: hydrolysis, %; abscissa: duration of incubation (hr). ○——○ *p*H 7; ×——× *p*H 7.4; ●——● *p*H 8.0.

into various buffer solutions was made to give a final concentration of 5.5 μg/ml. (2.13×10^{-5} M). 0.01 M-Citric acid/citrate buffer was used to obtain *p*H 3.05, 3.8, 4.9, 5.55 and 5.95, and 0.2 M-tris buffer for *p*H 6.8, 7.3, 7.6, 7.8 and 8.8 (Pye Cirscale *p*H meter at 20° C). These solutions were incubated at 37° C and samples were removed after 1 and 5 hr and the fall in extinction at 225 mμ was measured. Results are shown in Fig. 2.

In a second series of experiments the hydrolysis was followed more closely at *p*H 7, 7.4, 8 and 8.4 in 0.2 M-tris buffers; samples were removed at hourly intervals for determination of the optical density change. The amount of thalidomide hydrolysed was determined by referring the decrease in absorption at 225 mμ to the reference curve. The influence of *p*H on the hydrolysis of thalidomide is shown in Fig. 3.

Influence of temperature on buffer p*H*. Some of the buffer solutions used in this work were found to be markedly influenced by temperature changes. Thus the tris buffers of *p*H 7.0, 7.4, 8.0 and 8.4 at 19° C showed a reduction of 0.4 *p*H units when the temperature was raised to 37° C. Kolthoff phosphate buffer (0.067 M) was less influenced by temperature increase and buffers of *p*H 6.4, 6.8, 7.0 and 7.4 at 19° C increased in *p*H value by 0.1 unit. In practice the buffer solutions for incubation were prepared at room temperature (19 to 20° C) so that it should be borne in mind that for the tris buffers a decrease in *p*H value will have occurred when incubated at 37° C.

Hydrolysis of (+)- *and* (−)-*thalidomide*. The spontaneous rate of hydrolysis of (+)- and (−)-thalidomide has been compared with that of (±)-thalidomide at *p*H 7.2 and 18 and 27° C. All three substances were hydrolysed at very similar rates. The rate of hydrolysis was approximately 8%/hr and in 24 hr 80% of each isomer had decomposed.

Chromatography of thalidomide and its hydrolysis products

The chromatographic separation of thalidomide and its hydrolysis products was achieved using two solvent systems and Whatman No. 1 paper. The first solvent, A, consisted of pyridine—*n*-amyl alcohol—water in the ratio 7 : 7 : 6 by volume and the second, B, of *n*-butanol—glacial acetic acid, 10 : 1, saturated with water. Both were used by the descending technique, chromatograms running for 12 hr. After running in solvent A the paper was left for 12 hr to dry and then the paper was developed in solvent B; this solvent ran in a direction at right angles to that of solvent A. R_F values are given in Table 2.

Detection methods

Ultraviolet light. When the chromatograms were viewed in ultraviolet light (254 mμ) from a Hanovia " Chromatolite " lamp, spots consisting of thalidomide, 4-phthalimidoglutaramic acid and α-aminoglutarimide showed a greenish fluorescence after exposure of the paper for about 3 min. All the other compounds (see Table 2) showed up as dark spots on the background fluorescence of the paper, except α-(*o*-carboxybenzamido)-glutarimide which appeared as a dark purplish-blue spot. The exposure of thalidomide or 4-phthalimidoglutaramic acid to ultraviolet light appears to lead to the formation of a new substance with different chromatographic properties from its precursor.

Hydrazine spray. Chromatograms were sprayed with an ethanolic solution of hydrazine (5%) and then heated in an oven at 100° C for 10 min. The paper was then viewed under ultraviolet light as above. Under these conditions compounds containing the phthalimido

TABLE 2

CHROMATOGRAPHY OF THALIDOMIDE AND RELATED COMPOUNDS

The nature of solvents A and B and the procedures used are described in the text. The R_F values are multiplied by 100 and the ranges are given. The amount of each compound put on the paper was 20 μg. Methods of detection are given in the text. Green or blue colours indicate fluorescence

		R_F values × 100 in			Appearance of spot		
No.	Compound	A	B	Two-way system A ; B	In ultraviolet light	After ninhydrin spray	In ultraviolet light after hydrazine spray
I	Thalidomide	86–91	Streaks	86–91; 65–70	Dark, becoming green after 4–5 min exposure	—	Greenish-blue
II	4-Phthalimidoglutaramic acid	45–49	61–64	45–49; 53–58		—	Greenish-blue
III	2-Phthalimidoglutaramic acid	35–39	57–60	35–39; 45–51	Dark	—	Greenish-blue
IV	α-(*o*-Carboxybenzamido)glutarimide	36–39	61–65	36–39; 39–42	Purplish-black	Red developing slowly	Weak greenish-blue
V	2-Phthalimidoglutaric acid	24–29	80–86	24–29; 62–66	Dark	—	Greenish-blue
VI	4-(*o*-Carboxybenzamido)glutaramic acid	14–18	45–49	14–18; 24–27	Dark	—	Weak greenish-blue
VII	2-(*o*-Carboxybenzamido)glutaramic acid	15–19	47–52	15–19; 36–40	Dark	—	Weak greenish-blue
VIII	2-(*o*-Carboxybenzamido)glutaric acid	16–20	64–48	16–20; 43–47	Dark	—	Weak greenish-blue
IX	Phthalic acid	56–59	72–76	56–59; 58–61	Dark	—	Very weak blue
X	α-Aminoglutarimide	39–43	10–14	39–43; 9–12	Greenish	Red	None
XI	Isoglutamine	11–14	11–15	11–14; 8–11	Dark	Violet	None
XII	Glutamine	9–11	3– 7	9–11; 7– 7	Dark	Violet	None
XIII	Glutamic acid	4– 7	6–11	4– 7; 0–10	Dark	Violet	None

ring are readily converted into the greenish-blue fluorescent phthalazine-1,4-dione. Derivatives of phthalamic acid and phthalic acid do this less readily and show up as weak greenish-blue fluorescent spots.

Ninhydrin test. When the paper is sprayed with 0.5% ethanolic ninhydrin and then heated at about 80° C for 4 min the amino acids, glutamine, glutamic acid and isoglutamine, show up as purple spots, isoglutamine requiring longer heating than the other two. This spray also shows up α-aminoglutarimide as a reddish-purple spot and, since α-(*o*-carboxybenzamido)glutarimide decomposes to α-aminoglutarimide on heating, this compound is also revealed by this spray.

Identification of spontaneous hydrolysis products

Thalidomide. For the identification of the hydrolysis products, 15 mg of thalidomide was dissolved in warm acetone (1.5 ml.) and 0.067 M-Kolthoff-phosphate buffer, *p*H 7.4 (3.5 ml.), was added. The solution (*p*H 7.4) was filtered, and then incubated in stoppered tubes at 37° C for various periods. Small samples (0.1 to 0.5 ml.) were removed at intervals and chromatographed using the systems already described. Chromatography of the freshly prepared solution showed only thalidomide, indicating that the method of preparation of the solution was not responsible for any hydrolysis. After 4 hr incubation, eight spots apart from thalidomide were found using solvent A. At this stage, some of them could be identified. There were two ninhydrin positive spots indicating that complete hydrolysis to amino acids had occurred to some extent. 2- and 4-phthalimidoglutaramic acids, α-(*o*-carboxybenzamido)glutarimide and phthalic acid were identified by R_F values and colour reactions. The first three of these compounds are the primary hydrolysis products of thalidomide and could be expected to be formed first. Use of solvent B gave no further information.

After 24 hr incubation the use of solvents A and B and two-dimensional chromatography allowed the identification of all the possible hydrolysis products given in Fig. 1 with certainty. The compounds found and the range of R_F values by which they were identified are shown in Table 3.

TABLE 3

CHROMATOGRAPHY OF A SOLUTION OF THALIDOMIDE OF *p*H 7·4 AFTER KEEPING AT 37° C FOR 24 HR

The chromatogram was developed first in solvent A and then at right angles in solvent B, as described in the text

Compound detected	Final R_F values × 100	
	A	B
Thalidomide	82–89	60–70
4-Phthalimidoglutaramic acid	44–53	50–65
2-Phthalimidoglutaramic acid	34–40	45–60
α-(*o*-Carboxybenzamido)glutarimide	17–40	37–44
2-Phthalimidoglutaric acid	25–32	60–78
4-(*o*-Carboxybenzamido)glutaramic acid	9–18	20–30
2-(*o*-Carboxybenzamido)glutaramic acid	9–20	5–39
2-(*o*-Carboxybenzamido)glutaric acid	3–20	12–45
Phthalic acid	55–61	57–62
α-Aminoglutarimide	20–41	9–13
Isoglutamine	8–14	6–25
Glutamine	6–11	6–20
Glutamic acid	0– 6	0–24

The resolution obtained by two-dimensional chromatography is well illustrated in the case of the three free amino acids. When the solution of thalidomide, incubated for 24 hr, was chromatographed in solvent A, one amino acid spot was revealed by ninhydrin showing two areas of colouration more intense than the rest of the spot. These areas had R_F 0.04 and 0.08. On development at right angles with solvent B, the amino acid spot in A yielded three new spots corresponding to isoglutamine, glutamine and glutamic acid, the R_Fs of which are given in Table 3.

The chromatographic mobility of the compounds listed in Table 2 was influenced by a number of factors. These effects were checked using mixtures of the pure compounds. These factors accounted for the differences in R_F values for some of the compounds quoted in Table 3 as compared with the values in Table 2. These factors were:

(1) the amount of the compound applied to the paper;

(2) interaction between the compounds (for example, it was found that α-amino-glutarimide reacted with the free amino acids, resulting in multiple spot formation);

(3) interaction of some of the compounds with the solvents used in the buffer and the formation of salts with the pyridine of solvent A (salt formation with pyridine was noted particularly with the *o*-carboxybenzamido derivatives); and

(4) change in the nature of the paper during development of a chromatogram in solvent A so that subsequent R_F values using solvent B are different from those quoted in Table 2 for solvent B alone using the pure compounds.

Stability of the hydrolysis products of thalidomide

The stability of some of the hydrolysis products at various *p*H values and 37° C was examined spectrophotometrically and by paper chromatography.

Spectrophotometric method

For 2- and 4-phthalimidoglutaramic acids and 2-phthalimidoglutaric acid, which contain a phthalimide ring, the hydrolysis was followed spectrophotometrically by a method similar to that used for thalidomide.

A stock solution was prepared for each compound by dissolving 14.7 mg in 5 ml. of ethanol and diluting with water to 200 ml. A reference hydrolysis curve was prepared for each substance by treating 1.0, 2.0, 2.5, 3.0 and 4.0 ml. aliquots with 5 ml. of N-sodium hydroxide, neutralizing after 15 min with 5 ml. of N-hydrochloric acid and diluting to 50 ml. with 0.2 M-tris buffer of *p*H 7.4. The optical density of each solution at 225 mμ was read. A second series of aliquots were made up to 50 ml. with tris buffer after treatment with an equivalent volume of neutralized N-sodium hydroxide and extinctions at 225 mμ observed. A graph was constructed for each compound relating concentration to decrease in optical density at 225 mμ during hydrolysis. The extent of hydrolysis of the compound could be determined by measuring the decrease in optical density at 225 mμ, and referring this to the standard curve. The hydrolysis of each substance was measured in tris buffers of *p*H 6.0, 7.4 and 8.0 at 37° C using a concentration of 5.88 μg/ml. (2.13×10^{-5} M). Samples were withdrawn at hourly intervals and the extinction at 225 mμ was estimated. *In vitro* half-lives for 2- and 4-phthalimidoglutaramic acid and 2-phthalimidoglutaric acid are shown in Table 4.

TABLE 4

IN VITRO HALF-LIVES OF THALIDOMIDE AND ITS HYDROLYSIS PRODUCTS AT VARIOUS *p*Hs

Substances were incubated at 37° C in tris buffers of *p*H 6, 7, 7·4 and 8 at a concentration of $2{\cdot}13\times10^{-5}$ M. The hydrolysis of the phthalimide ring was followed by observing the fall in extinction at 225 mμ. * Not determined

Compound	Half-life (hr) at *p*H			
	6	7	7·4	8·0
Thalidomide	—*	11	5	1·25
2-Phthalimidoglutaramic acid	48	—*	48	30
4-Phthalimidoglutaramic acid	24	—*	5	2
2-Phthalimidoglutaric acid	48	—*	43	33

Chromatographic method

Solutions of each substance studied were prepared by dissolving about 15 mg of the compound in 0.067 M-Kolthoff-phosphate buffer, *p*H 7.4, and incubating at 37° C. Aliquots (0.1 ml.) were withdrawn at 1-, 4- and 24-hr intervals and used for two-dimensional paper chromatography in the solvents already described.

4-Phthalimidoglutaramic acid. This substance was particularly unstable, hydrolysis of the phthalimide ring readily occurring at *p*H 6 and the rate of hydrolysis was accelerated markedly by raising the *p*H. At *p*H 6 the half-life was about 24 hr and this decreased to 5 hr at *p*H 7.4 and 2 hr at *p*H 8. Chromatography of the three incubated solutions showed that 4-(*o*-carboxybenzamido)glutaramic acid was the major hydrolysis product.

2-Phthalimidoglutaramic acid. This substance was much more stable to ring hydrolysis than its 4-isomer. Thus at *p*H 8 its half-life was about 30 hr, while at *p*H 7.4 and 6 less than a third had decomposed after 48 hr incubation. However, chromatography revealed that, while the major hydrolysis product was 2-(*o*-carboxybenzamido)glutaramic acid, some hydrolysis of the α-amide group to give 2-phthalimidoglutaric acid had occurred. In this respect, 2-phthalimidoglutaramic acid differed from its 4-isomer in which the γ-amide group was stable to hydrolysis under the conditions of the experiment.

2-Phthalimidoglutaric acid. At *p*H 7.4 and 8.0 the half-lives of this compound were 43 and 33 hr respectively. At *p*H 6 the substance was very stable and the half-life was of the order of 4 days. The major hydrolysis product was 2-(*o*-carboxybenzamido)glutaric acid.

2- and 4-(o-carboxybenzamido)glutaramic acid and 2-(o-carboxybenzamido)glutaric acid. These three compounds on incubation at *p*H 7.4 and 37° C appeared to be more stable than any of the preceeding compounds used in this study. In 4 hr there was no detectable decomposition. After 24 hr only traces of free amino acids and no phthalic acid could be detected on chromatograms. The main component of the incubated solution was the original compound.

α-Aminoglutarimide. After incubating this compound for 1 hr, chromatograms showed traces of amino acids. After 8 hr, the unchanged compound and amino acids were readily detected, together with two unknown substances which on paper quenched ultraviolet light.

After 24 hr, α-aminoglutarimide had almost completely disappeared and the chromatograms showed the presence of isoglutamine, glutamine and glutamic acid together with three unknown spots. Two of these spots appeared as dark areas on chromatograms when

viewed in ultraviolet light. They also gave a pink colour with ninhydrin. The third unknown spot was dark blue in colour and did not react with ninhydrin. On keeping an aqueous solution of α-aminoglutarimide at room temperature for 24 hr it turns blue, as does a drop of the solution put on a filter paper. This blue compound is probably an oxidation product, the nature of which is at present unknown (see Sondheimer & Holley, 1957).

α-(o-*Carboxybenzamido)glutarimide.* This compound was stable below *p*H 6 but at *p*H 6 to 7 the substance was slowly hydrolysed to α-aminoglutarimide and phthalic acid and to traces of 2- and 4-(*o*-carboxybenzamido)glutaramic acids. At *p*H 7.4, the hydrolysis was more rapid and, besides the unchanged compound, phthalic acid, α-aminoglutarimide, isoglutamine, glutamine, glutamic acid, an unidentified blue compound and 2- and 4-(*o*-carboxybenzamido)glutaramic acids were detected in the incubated solution.

Reference to Fig. 1 shows that there are three primary hydrolysis products, namely compounds II, III and IV. α-(*o*-Carboxybenzamido)glutarimide (IV) is formed by splitting of either of the equivalent amide bonds of the phthalimide ring, and 4- and 2-phthalimidoglutaramic acids (II and III) by splitting of one or the other amide link of glutarimide. The splitting of these three links may have different *p*H sensitivities. Thalidomide solutions were therefore incubated at *p*H 6.0, 6.5, 6.8, 7.0 and 7.4 in 0.067 M-Kolthoff-phosphate buffers for 4 hr and the solutions were examined chromatographically. At the lower *p*Hs (6.0 to 6.5) thalidomide was converted into α-(*o*-carboxybenzamido)glutarimide (IV), but a *p*H above 6.8 to 7.0 was needed to form 4- and 2-phthalimidoglutaramic acids (II and III). Thus the amide bonds of the phthalimide ring could be split at *p*H 6 to 7, whereas a *p*H of over 7 was needed to split the amide bonds of the glutarimide ring.

Quantitative aspects of the hydrolysis of thalidomide

The quantitative aspects of the formation of the various hydrolysis products has been studied at *p*H 6, 7.4 and 8 using (±)-α-{(1-[^{14}C]-carbonyl)phthalimido}glutarimide synthesized from (1-[^{14}C]-carbonyl)phthalic anhydride (Beckmann, 1962). Aliquots (0.1 ml.)

TABLE 5
EFFECT OF *p*H ON THE HYDROLYSIS OF THALIDOMIDE

	Amount of hydrolysis product formed (%) at								
	*p*H 6			*p*H 7·4			*p*H 8		
Compound	1 hr	4 hr	24 hr	1 hr	4 hr	24 hr	1 hr	4 hr	24 hr
α-(*o*-Carboxybenzamido)glutarimide	4·2	12·7	34·5	23·1	47·5	52·5	41·3	58·0	14·0
4-Phthalimidoglutaramic acid	0·7	2·5	4·9	1·6	3·5	2·0	16·0	3·7	3·3
2-Phthalimidoglutaramic acid	0	0	1·3	0·3	1·1	1·8	2·3	2·8	6·2
2-Phthalimidoglutaric acid	0	0	0	0	0	0·1	0·7	1·0	2·7
4-(*o*-Carboxybenzamido)glutaramic acid	0	0	0·7	0·3	1·8	23·0	2·9	18·0	40·5
2-(*o*-Carboxybenzamido)glutaramic acid	0	0	0	0·3	1·6	20·0	1·8	10·0	25·7
2-(*o*-Carboxybenzamido)glutaric acid	0	0	0	0	0	0·1	0·2	0·5	5·2
Phthalic acid	0	0	0	0	0	0·1	0·4	1·4	1·9
Thalidomide	95·1	84·0	58·3	71·5	41·5	0·15	34·0	3·2	0·03
Total	99·9	99·2	99·7	96·7	97·0	99·8	99·6	98·6	99·7
Thalidomide hydrolysed	4·9	15·2	44·4	28·3	58·5	99·8	66·0	96·8	99·9

of 1-, 4- and 24-hr incubated solutions were chromatographed using the two-dimensional technique and the various hydrolysis products resolved. Areas containing the compounds were cut out (usually as a 2×2-cm square) and the radioactivity was determined using a scintillation spectrometer (Packard Model 3214). The scintillation solution contained 5 g/l. of 2,5-diphenyloxazole and 0.3 g/l. of 1,4-bis(5-phenyloxazolyl)benzene in toluene. The total radioactivity on the paper was estimated, corrected for background and the radioactivity associated with each hydrolysis product was calculated as a percentage of the total radioactivity.

Preparation of incubated solution. About 10 mg of [^{14}C]-thalidomide was shaken with 5 ml. of the appropriate buffer solution for 3 min, filtered, and the filtrate was incubated at 37° C for 24 hr. Aliquots (0.5 ml.) were removed at 1, 4 and 24 hr and chromatographed. Buffer solutions consisted of Kolthoff 0.067 M-phosphate buffer of *p*H 6, 7.4 and 8.

The influence of *p*H on the formation of the various hydrolysis products of thalidomide is shown in Table 5.

DISCUSSION

The experiments described in this paper show that thalidomide is unstable in aqueous solution at physiological *p*H values and that any solution of thalidomide of *p*H greater than 7.0 even after 1 hr is likely to contain up to twelve other compounds besides thalidomide. If thalidomide is absorbed as such it is likely that in the blood it will gradually hydrolyse spontaneously into compounds which are on the whole more soluble than the original drug. Any study of the pharmacological and biochemical properties of thalidomide must take this instability into account. It is to be noted that any solution made by dissolving thalidomide in alkali such as 0.1 N-sodium hydroxide contains no thalidomide since this compound is immediately hydrolysed by this strength of alkali and is not reformed on acidification.

Our chromatographic studies show that thalidomide is hydrolysed in stages as expected from Fig. 1. The primary products are 4-phthalimidoglutaramic (II), 2-phthalimidoglutaramic acid (III) and α-(*o*-carboxybenzamido)glutarimide (IV), each of these being formed by the hydrolysis of a different amide link in the thalidomide molecule.

At *p*H 6 thalidomide is slowly hydrolysed and the main product of the hydrolysis is α-(*o*-carboxybenzamido)glutarimide (IV) with only small amounts of (II) and (III) produced by cleavage of the glutarimide ring. At physiological *p*H of 7.4, thalidomide is more rapidly hydrolysed; 28% disappeared in 1 hr and practically 100% in 24 hr. At this *p*H large amounts of secondary hydrolysis products appear, particularly 2- and 4-(*o*-carboxybenzamido)glutaramic acids (VI and VII). At this *p*H the glutarimide ring is susceptible to hydrolysis to give 2- and 4-phthalimidoglutaramic acids (II and III). However, compound (II) is very unstable at *p*H 7.4 (half-life 5 hr) and it readily breaks down to 4-(*o*-carboxybenzamido)glutaramic acid (VI). At *p*H 8 the hydrolysis is very rapid; 66% of the drug is hydrolysed in 1 hr. The main product formed is, once again, compound IV but larger amounts of compound II are formed, but at this *p*H 4-phthalimidoglutaramic acid has a half-life of 2 hr and it is rapidly cleaved to compound VI. While an increase in *p*H from 7.4 to *p*H 8 doubles the rate of hydrolysis of the phthalimido ring, the rate of cleavage of the glutarimide ring is increased more than tenfold.

The main route of hydrolysis of thalidomide at *p*H 6, 7.4 and 8 is cleavage of the phthalimido ring to α-(*o*-carboxybenzamido)glutarimide (IV). This compound is reasonably stable at these *p*H values, however, and its main products on hydrolysis are 2- and 4-(*o*-carboxybenzamido)glutaramic acids. A small portion is hydrolysed to phthalic acid (IX) and α-aminoglutarimide (X) and this latter substance is further hydrolysed to isoglutamine (XI), glutamine (XII) and glutamic acid (XIII).

As the *p*H is increased the bonds of the glutarimide ring become susceptible to hydrolysis and, at *p*H 7.4 and 8 especially, considerable amounts of 2- and 4-phthalimidoglutaramic acids are formed. The origin of 2-phthalimidoglutaric acid is probably I⟶III⟶V, since the γ-amide group of 2-phthalimidoglutaramic acid is more easily hydrolysed than the α-amide group of its corresponding 4-isomer (II).

These studies suggest that, owing to the lability of thalidomide and its primary hydrolysis products, a buffered solution of thalidomide at *p*H 7 to 8 will contain within a period of 24 hr all the compounds listed in Fig. 1. In the following paper (Schumacher *et al.*, 1965) it will be shown that this situation also obtains in the tissues, blood and urine of animals dosed with thalidomide.

SUMMARY

1. When thalidomide is administered to laboratory animals, there are excreted in the urine, besides small amounts of the unchanged compound, some twelve substances which are derived from the parent drug by simple hydrolysis. This suggests that thalidomide may be inherently unstable and may undergo spontaneous hydrolysis in the body giving rise to all twelve of its possible hydrolysis products.

2. The spontaneous hydrolysis of thalidomide at various *p*H values was followed spectrophotometrically and the hydrolysis products formed were identified by their R_F values and colour reactions on paper chromatograms. The amount of each individual hydrolysis product formed in buffers at *p*H 6, 7.4 and 8.0 was estimated using [^{14}C]-thalidomide. The hydrolysis products present in an incubated solution of [^{14}C]-thalidomide were resolved on a two-dimensional chromatogram and the radioactivity associated with each compound was estimated using a scintillation spectrometer.

3. At *p*H values above 6, thalidomide undergoes spontaneous hydrolysis; the rate at which this occurs accelerates with increase in *p*H. At *p*H 7, 7.4 and 8 thalidomide has a half-life of 11, 5 and 1.25 hr respectively. All the substituted amide bonds of the thalidomide molecule are sensitive to hydrolysis and, at *p*H 7.4, all twelve possible hydrolysis products are formed by splitting of these groups. At all *p*H values used the main hydrolysis product is α-(*o*-carboxybenzamido)glutarimide but at *p*H values above 7 increasing amounts of 2- and 4-phthalimidoglutaramic acids are formed. These three primary hydrolysis products are also unstable and can undergo further secondary, tertiary and quaternary hydrolyses.

4. The various substituted amide bonds possess different sensitivities to hydroxyl ion-catalysed hydrolysis. From *p*H 6 to 7, only the phthalimide ring undergoes cleavage, whereas at *p*H 7 and above the glutarimide moiety also suffers hydrolytic splitting.

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