Chiral Inversion and Hydrolysis of Thalidomide: Mechanisms and Catalysis by Bases and Serum Albumin, and Chiral Stability of Teratogenic Metabolites

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The chiral inversion and hydrolysis of thalidomide and the catalysis by bases and human serum albumin were investigated by using a stereoselective HPLC assay. Chiral inversion was catalyzed by albumin, hydroxyl ions, phosphate, and amino acids. Basic amino acids (Arg and Lys) had a superior potency in catalyzing chiral inversion compared to acid and neutral ones. The chiral inversion of thalidomide is thus subject to specific and general base catalysis, and it is suggested that the ability of HSA to catalyze the reaction is due to the basic groups of the amino acids Arg and Lys and not to a single catalytic site on the macromolecule. The hydrolysis of thalidomide was also base-catalyzed. However, albumin had no effect on hydrolysis, and there was no difference between the catalytic potencies of acidic, neutral, and basic amino acids. This may be explained by different reaction mechanisms of the chiral inversion and hydrolysis of thalidomide. Chiral inversion is deduced to occur by electrophilic substitution involving specific and general base catalysis, whereas hydrolysis is thought to occur by nucleophilic substitution involving specific and general base as well as nucleophilic catalysis. As nucleophilic attack is sensitive to steric properties of the catalyst, steric hindrance might be the reason albumin is not able to catalyze hydrolysis. ^{1}H NMR experiments revealed that the three teratogenic metabolites of thalidomide, in sharp contrast to the drug itself, had complete chiral stability. This leads to the speculation that, were some enantioselectivity to exist in the teratogenicity of thalidomide, it could result from fast hydrolysis to chirally stable teratogenic metabolites.

Introduction

Thalidomide [α-(*N*-phthalimido)glutarimide], originally introduced as a non-barbiturate sedative with antinausea properties, was withdrawn from the market in 1961 because of its catastrophic teratogenicity (*1*, *2*). Since Sheskin (*3*) reported that thalidomide caused a dramatic improvement of the inflammatory reactions of leprosy patients, clinical interest in thalidomide began to rise again. It has become widely employed in the treatment of erythema nodosum leprosum and has also been successfully used to treat various inflammatory conditions and autoimmune diseases (*4*−*7*). Besides sedative and neurotropic effects, teratogenicity, neurotoxicity, and anti-inflammatory and immunomodulatory activity, thalidomide has also been reported to reduce the rate of HIV[1] type 1 replication in vitro (*8*, *9*). Although the mechanisms of action of thalidomide are only poorly understood, some of its activities may be related to its capacity to inhibit the production of TNF-α (*10*).

In all medical treatments thalidomide, which has one chiral center, is administered in the form of a racemate. When the fact that enantiomers can have very different pharmacological activities is considered (*11*, *12*), the question of whether the biological activities of thalidomide are associated with either one or both of the enantiomers arises. Unfortunately, findings in the literature are contradictory, and it is presently still unclear whether any of the effects of thalidomide are enantioselective (*13*). The problem is complicated by the fact that the enantiomers of thalidomide are subject to rapid chiral inversion in vitro (*14*) and in vivo (*15*). In vitro studies showed that the chiral inversion of thalidomide is catalyzed by human serum albumin (HSA) and that this catalysis can be inhibited by various albumin ligands such as long- and medium-chain fatty acids and acetylsalicylic acid (*14*, *16*). However, no detailed mechanism of the chiral inversion of thalidomide and its catalysis by HSA has been suggested.

Thalidomide has been found to be eliminated mainly by spontaneous hydrolysis in blood and tissues of humans and animals (*17*, *18*). All four amide bonds of the molecule are susceptible to hydrolytic cleavage at pH >6 (*19*) (Figure 1). The main urinary metabolites in humans are 2-phthalimidoglutaramic acid (**3**) (about 50%) and α-(o-carboxybenzamido)glutarimide (**4**) (about 30%) (*20*). Of the 12 hydrolysis products of thalidomide, only those three which contain the intact phthalimide moiety showed teratogenic activity, i.e., 2- (**3**) and 4-phthalimidoglutaramic acid (**2**) and 2-phthalimidoglutaric acid (**5**) (*21*, *22*) (Figure 1). An investigation of the teratogenic potency of the optical antipodes of 2-phthalimidoglutaric

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[1] Abbreviations: HIV, human immunodeficiency virus; HSA, human serum albumin; TNF-α, tumor necrosis factor-α; ASA, acetylsalicylic acid.

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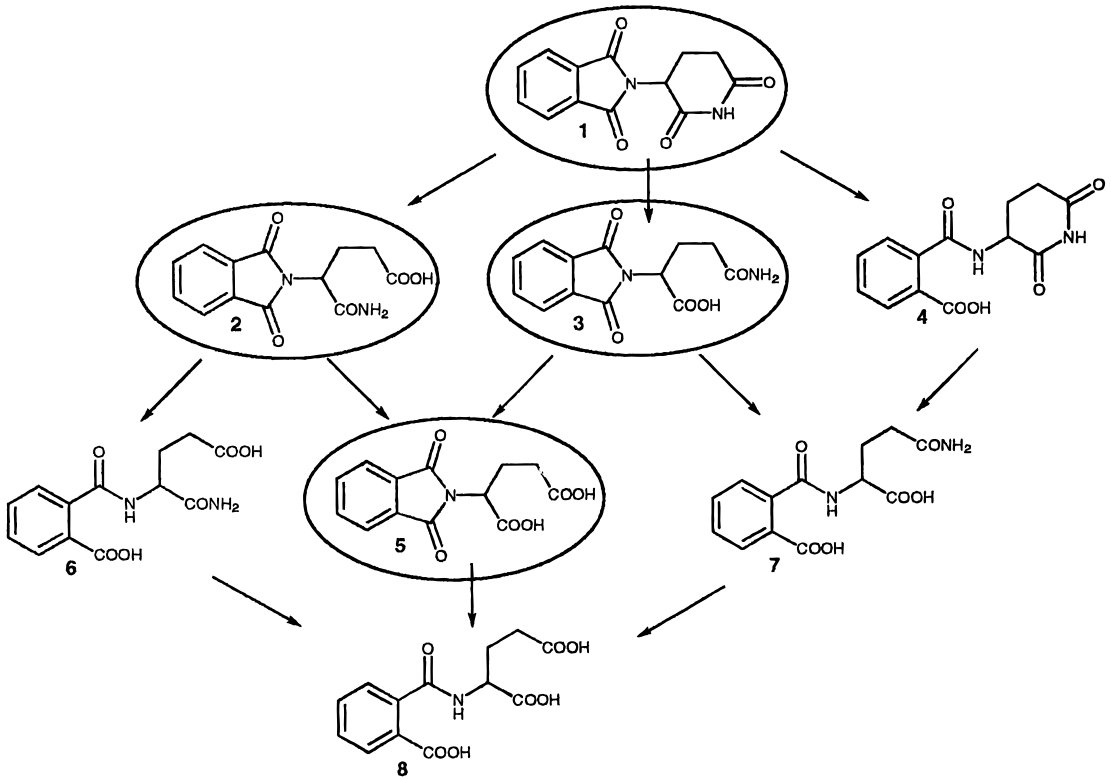

Figure 1. First products in the hydrolytic degradation of thalidomide in aqueous solutions at pH >6: thalidomide (**1**), 4-phthalimidoglutaramic acid (**2**), 2-phthalimidoglutaramic acid (**3**), α-(*o*-carboxybenzamido)glutarimide (**4**), 2-phthalimidoglutaric acid (**5**), 4-(*o*-carboxybenzamido)glutaramic acid (**6**), 2-(*o*-carboxybenzamido)glutaramic acid (**7**), and 2-(*o*-carboxybenzamido)glutaric acid (**8**). Teratogenic compounds are circled (modified from ref *19*).

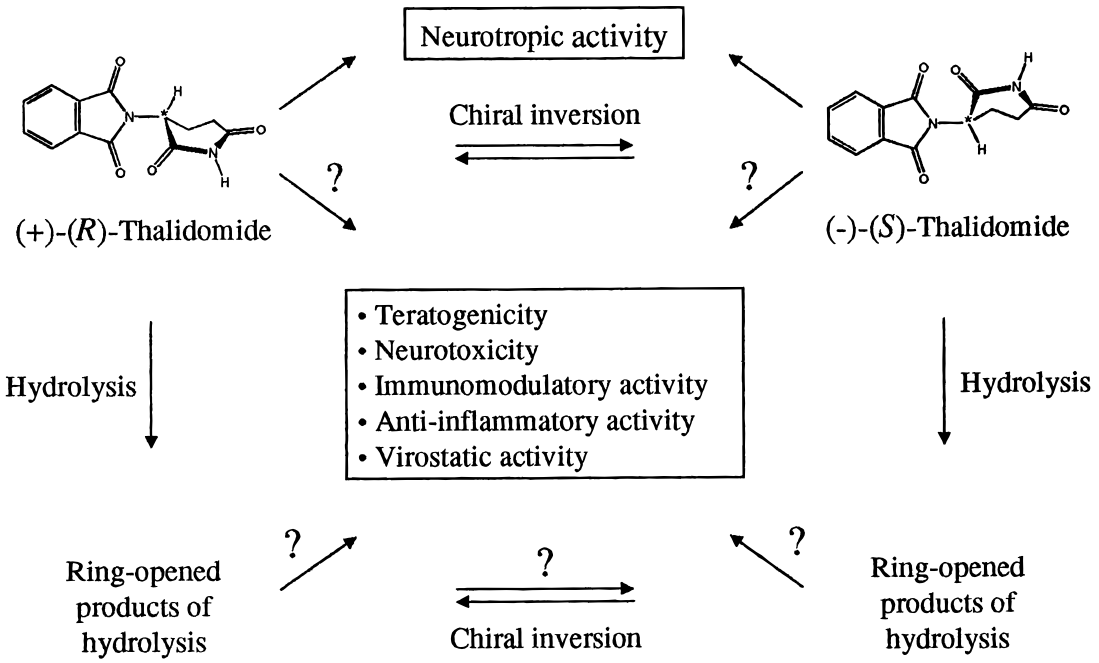

Figure 2. Interplay of biological activities, metabolism, and stereoselectivity of thalidomide enantiomers and their ring-opened metabolites.

acid (**5**) in pregnant mice showed that the *S*-enantiomer caused dose-dependent teratogenicity, whereas the *R*-enantiomer was devoid of an effect even at doses 4 times higher (*23*, *24*).

In Figure 2, the biological activities of thalidomide are summarized, and these data hint at the complexity of its metabolism and activities. Some of the questions emphasized in Figure 2 can be answered in part. Others, however, do not appear to have been adequately investigated. For example, there is no information available about the configurational stability of the three teratogenic metabolites of thalidomide. In this paper, the mechanism of chiral inversion of thalidomide and its catalysis by HSA and general bases was investigated by using a stereoselective HPLC assay. Further, the configurational stability of the teratogenic metabolites of thalidomide was

established, and implications on the stereoselectivity of the teratogenicity of thalidomide are discussed.

Materials and Methods

Chemicals. (−)-(*S*)-Thalidomide [(−)-(*S*)-1,3-dioxo-2-(2′,6′-dioxopiperidin-3′-yl)isoindol] and (+)-(*R*)-thalidomide were obtained by preparative chromatographic separation on Chiralcel OJ. The preparative HPLC was performed with a Shimadzu modular liquid chromatograph (Burkhardt Instrumente, Zürich, Switzerland) composed of a LC-8A pump and a multiwavelength UV/Vis model SPD-10A detector. The UV signal was recorded and processed by an Epson microcomputer, using the Class LC-10 chromatographic software (Shimadzu, Burkhardt Instrumente). Racemic thalidomide (500 mg) prepared according to a reported method (*25*) was dissolved in 25 mL of dichloromethane and diluted with 500 mL of ethanol. This solution was injected via the pump on a 5 cm (i.d.) × 50 cm Chiralcel OJ column (Daicel Chemical Industries). Chromatography was carried out at room temperature at a flow rate of 150 mL/min, and UV detection was performed at 220 nm. The mobile phase was pure ethanol containing small amounts of trifluoroacetic acid (0.05%) to avoid racemization of the isolated enantiomers during workup. Under the applied chromatographic conditions, the *R*-enantiomer was isolated from a first fraction collected between 38 and 50 min and the *S*-enantiomer from a second fraction collected between 58 and 80 min. The enantiomeric purity of the isolated enantiomers, determined by chiral HPLC as described in Chromatographic Conditions, was 99.70% for (*S*)-thalidomide and 99.92% for (*R*)-thalidomide.

(*RS*)-2-Phthalimidoglutaramic acid (phthalyl-DL-glutamine) (**3**) and (*RS*)-4-phthalimidoglutaramic acid (phthalyl-DL-isoglutamine) (**2**) were prepared from L-glutamic acid and phthalic anhydride according to the method described by King et al. (*26*, *27*) and recrystallized from water or aqueous alcohol, respectively. The identity and purity of the two substances were confirmed by melting points and ^{1}H NMR spectra.

HSA (fraction V, essentially fatty acid-free, A-1887, lot 42H9313) was purchased from Sigma (Buchs, Switzerland). Anthracene *puriss*., (*S*)-2-phthalimidoglutaric acid (*N*-phthaloyl-L-glutamic acid) (**5**), glycine, L-serine, L-lysine monohydrochloride, L-arginine monohydrochloride, L-aspartic acid, L-glutamic acid, L-glutamine, phthalic anhydride, potassium chloride, potassium dihydrogen phosphate, and disodium hydrogen phosphate anhydrous were obtained from Fluka Chemie AG (Buchs, Switzerland) and ethanol absolute, ethyl acetate, and acetonitrile, all being HPLC quality, from Romil Chemicals (Cambridge, England). The deuterated solvents were purchased from Armar (Döttingen, Switzerland), and their isotopic purities were 99.8 at. % D D_2O, >99.5 at. % D sodium deuterium oxide (40%) in D_2O, and 99.8 at. % D DMSO-d_6. All other chemicals and solvents were obtained from Fluka Chemie AG or Merck (Darmstadt, Germany); they were of analytical grade and used without further purification.

Influence of Albumin, pH, Phosphate, and Amino Acids on the Chiral Inversion and Hydrolysis of Thalidomide by HPLC. (1) Chromatographic Conditions. The HPLC system consisted of a model 360 autosampler, a model 420 pump, a model 440 diode array detector, a model 450 MT2/DAD data system, a model 800 plotter (all from Kontron Instruments, Zürich-Müllingen, Switzerland). The thalidomide enantiomers and the internal standard anthracene were separated on a 250 mm × 4 mm column with *p*-methylbenzoylcellulose coated on silica as the stationary phase (*28*). The mobile phase was absolute ethanol (Romil Chemicals), the flow rate 0.7 mL/min, and the detection wavelength 230 nm.

(2) Preparation of Buffers and Solutions. Stock solutions of (*R*)- and (*S*)-thalidomide were prepared weekly in acetonitrile (0.5 mg/mL, 1.94×10^{-3} M), and the internal standard (anthracene) was dissolved daily in ethyl acetate (0.1 mg/mL, 5.61×10^{-4} M). HSA solutions (HSA fraction V, essentially fatty acid-free, Sigma A-1887) at nine different concentrations (5.0×10^{-7}, 2.0×10^{-6}, 1.0×10^{-5}, 4.0×10^{-5}, 7.46×10^{-5}, 1.49×10^{-4}, 1.80×10^{-4}, 2.99×10^{-4}, and 5.97×10^{-4} M) in phosphate buffer (0.1 M, pH 7.40, ionic strength of 0.3) were prepared to study the influence of HSA concentration. To investigate the influence of pH, 0.1 M phosphate buffers (Sørensen) at six different pH values (2.03, 3.58, 6.06, 7.44, 8.03, and 11.00) and an ionic strength of 0.3 were prepared. To study the influence of phosphate concentration, phosphate buffers (Sørensen) at four different concentrations (0.05, 0.10, 0.20, and 0.30 M) with a constant pH of 7.40 and a final ionic strength of 0.8 were prepared. The influence of neutral, acidic, and basic amino acids was investigated in 0.1 M solutions of amino acids Arg, Asp, Gln, Glu, Gly, Lys, and Ser in phosphate buffer (0.1 M, pH 7.40, ionic strength of 0.3) with a constant pH of 7.40 and a final ionic strength of 0.9. All solutions were freshly prepared.

(3) Incubation Conditions. Fifty microliters of the stock solution of the enantiomers was evaporated to dryness under a stream of nitrogen. The final concentration of each enantiomer in the reaction mixture was about 1×10^{-4} M. The reaction was started by adding 1 mL of incubation medium (solutions described above), and the mixture was kept in a rotating water bath (Infors HT WTR1, Infors AG, Bottmingen, Switzerland) at 37 ± 0.2 °C. At various time intervals, the reaction was stopped by immediate extraction as described below.

(4) Extraction. After addition of 50 μL of the anthracene solution as an internal standard, the incubation mixtures were extracted with 4 mL of ethyl acetate for 10 min. The organic layers were separated by centrifugation (1500*g* for 10 min). Three milliliters of the organic layers was transferred to a tube, and the solvent was then evaporated to dryness under vacuum and a stream of nitrogen. The residues were dissolved in 300 mL of absolute ethanol, and 20 μL aliquots were injected onto the column.

For the validation of the extraction, solutions of anthracene and *rac*-thalidomide at known concentrations and solutions of mixtures of the pure thalidomide enantiomers with known enantiomeric ratios were prepared in 99.8% ethanol. An aliquot of these solutions was evaporated to dryness under a stream of nitrogen. One milliliter of phosphate buffer (0.30 M, pH 7.40, ionic strength of 0.8) or HSA solution (5.0×10^{-7}, 2.0×10^{-6}, 1.0×10^{-5}, 4.0×10^{-5}, and 5.97×10^{-4} M) was added and the extraction continued as described above.

(5) Estimation of Rate Constants. In the case of nonstereoselective inversion of configuration in phosphate buffers and amino acid solutions, the observed pseudo-first-order rate constants of enantiomerization or racemization (*29*) were obtained by plotting the natural logarithm of the decreasing enantiomeric excess of the predominating enantiomer as a function of time according to eq 1:

$$\ln\left(\frac{[E_{dec}]_t - [E_{inc}]_t}{[E_{dec}]_t + [E_{inc}]_t}\right) = -k_{rac}t = -2k_{enant}t \qquad (1)$$

where $[E_{dec}]_t$ is the concentration of the enantiomer whose level is decreasing at time t, $[E_{inc}]_t$ the concentration of the enantiomer whose level is increasing at time t, k_{rac} the observed pseudo-first-order rate constant of racemization, and k_{enant} that of enantiomerization.

In the case of a stereoselective inversion of configuration, i.e., in albumin solutions, the observed pseudo-first-order rate constants of chiral inversion $k_{(R\text{-to-}S)}$ and $k_{(S\text{-to-}R)}$ were estimated by nonlinear regression analysis of the experimentally determined, decreasing weighted difference of the two enantiomers, using eq 2. The nonlinear regression analyses were performed with GraphPad-Prism 1.03 (GraphPad Software, Sorrento Valley, San Diego, CA)

$$\frac{[E_{dec}]_t - [E_{inc}]_t}{[E_{dec}]_t + [E_{inc}]_t} = \frac{k_2 - k_1}{k_1 + k_2} + 2\frac{k_1}{k_1 + k_2}e^{-(k_1+k_2)t} \qquad (2)$$

where k_2 is the observed pseudo-first-order rate constant of chiral inversion of the *R*-enantiomer to the *S*-enantiomer,

$k_{(R\text{-to-}S)}$, and k_1 that of the chiral inversion of the *S*-enantiomer to the *R*-enantiomer, $k_{(S\text{-to-}R)}$.

The observed pseudo-first-order rate constants of hydrolysis were obtained by plotting the natural logarithm of the decreasing concentration of thalidomide as a function of time according to eq 3:

$$\ln\left(\frac{[\text{Thalid}]_t}{[\text{Anthra}]_t}\right) = -k_{\text{hyd}}t \quad (3)$$

where $[\text{Thalid}]_t$ is the total thalidomide concentration at time *t*, $[\text{Anthra}]_t$ the concentration of the internal standard anthracene at time *t*, and k_{hyd} the observed pseudo-first-order rate constant of hydrolysis.

For four albumin concentrations, the observed pseudo-first-order rate constants of chiral inversion $k_{(R\text{-to-}S)}$ and $k_{(S\text{-to-}R)}$ as well as those of hydrolysis were estimated by fitting of a two-compartment kinetic model with elimination from both compartments to the measured concentrations of (*R*)- and (*S*)-thalidomide during incubation of either enantiomer. Model equations were written with Siphar software (Siphar/PC Version 4.0, Simed S.A., Créteil, France), and the model was fitted simultaneously to the concentration curves of (*R*)- and (*S*)-thalidomide obtained in each experiment.

Investigation of the Configurational Stability and Hydrolysis of the Three Teratogenic Thalidomide Metabolites by ^{1}H NMR. The configurational stability and hydrolysis of the three teratogenic thalidomide metabolites **2**, **3**, and **5** were monitored by ^{1}H NMR (^{1}H/^{2}H substitution). The method takes advantage of the fact that the inversion of chiral centers of the type R″R′RCH and proton−deuterium exchange share a common mechanism (*30*−*33*). All ^{1}H NMR spectra and integrals were recorded on a Varian VXR-200 NMR spectrometer operating at 200 MHz (Varian Associates Inc., Palo Alto, CA). 4-Phthalimidoglutaramic acid (**2**), 2-phthalimidoglutaramic acid (**3**), or 2-phthalimidoglutaric acid (**5**) (25 mg) was dissolved in 0.8 mL of phosphate buffer (0.3 M, pD 7.4) and the pD value adjusted to 7.4 with 1 M NaOD. The pD values were measured at room temperature (25 °C) using a Metrohm 654 pH-meter (Herisau, Switzerland) coupled with a glass electrode and calibrated with standard aqueous buffers. The pD values were obtained by adding a correction factor of 0.4 to the measured pH values (*34*, *35*). Each such solution was placed in a sealed NMR tube and each tube kept in a water bath (Haake D1, Haake GmbH, Karlsruhe, Germany) at 37 °C. At various time intervals of up to 7 days, ^{1}H NMR spectra and integrals were recorded at room temperature. Deuteration [i.e., chiral inversion or racemization (*36*)] and hydrolysis can be observed by following changes in the NMR spectra. Deuteration was monitored by following the decrease of the sum of the integral of the signal of the proton coupled to the chiral center of the thalidomide metabolites (**2**, **3**, and **5**) and the integral of the signal of the proton coupled to the chiral center of the hydrolyzed metabolites (i.e., **6**−**8**), in relation to the integral of the signal of the four aliphatic protons in the α- and *β*-positions with respect to the chiral center (unresolved bulk resonances). The observed pseudo-first-order rate constants of hydrolysis were estimated by plotting the natural logarithm of the increasing integral of the signal of the proton coupled to the chiral center of the hydrolyzed metabolites (i.e., **6**−**8**) as a function of time according to eq 4:

$$\ln(C_t/B_t) = k_{\text{hyd}}t \quad (4)$$

where C_t is the integral of the proton coupled to the chiral center of the hydrolyzed metabolites at time *t* and B_t the integral of the unexchangeable reference protons (i.e., the four aliphatic protons in the α- and *β*-positions with respect to the chiral center) at time *t*.

Results

Validation of the Stereospecific HPLC Assay Used To Investigate the Chiral Inversion and Hydrolysis of Thalidomide. The extraction method used in this study was similar to that reported by Chen et al. (*14*, *18*). After extraction of the incubation mixtures with ethyl acetate, three peaks were seen in the chromatograms, one for each enantiomer of thalidomide and the third for the internal standard anthracene. The resolution factor of (*R*)-thalidomide (t_R = 7.86 min) versus (*S*)-thalidomide (t_R = 13.43 min) was 1.5, and the resolution factor of (*S*)-thalidomide versus anthracene (t_R = 18.36 min) was 1.3. Due to their anionic structure at pH 7.4, the products of hydrolysis of thalidomide were not extracted.

(1) Linearity between *rac*-Thalidomide and the Internal Standard Anthracene before and after Extraction. Anthracene proved to be a suitable internal standard. Solutions with eight different concentrations of anthracene (between 7.0×10^{-5} and 1.4×10^{-3} M) and a constant concentration of *rac*-thalidomide and solutions with eight different concentrations of *rac*-thalidomide (from 4.8×10^{-5} to 9.7×10^{-4} M) and a constant concentration of anthracene were analyzed by HPLC before and after extraction from phosphate buffer (0.30 M, pH 7.40, ionic strength of 0.8) or HSA solutions with four different concentrations. Linear relationships between thalidomide and anthracene concentrations were found before and after extraction from the different incubation media ($r^2 > 0.995$). The extraction yields of (*R*)- and (*S*)-thalidomide were 92.8 ± 6.8 and 92.5 ± 7.2%, respectively (mean ± SD).

(2) Validation of Enantiomeric Ratios before and after Extraction. Thalidomide solutions with eleven different enantiomeric ratios (*R*/*S* ratio between 0.1 and 10.0) were analyzed by HPLC before and after extraction from phosphate buffer (0.30 M, pH 7.40, ionic strength of 0.8) or HSA solutions with four different concentrations. The relation between the relative peak areas determined by HPLC in percent and the percentage of enantiomer injected was linear for all determinations (r^2 = 0.999).

Influence of HSA Concentration on the Chiral Inversion and Hydrolysis of Thalidomide. Apparent pseudo-first-order rate constants of chiral inversion and hydrolysis (k_{hyd}) were determined in a series of fatty acid-free HSA solutions over a concentration range of 5.0×10^{-7} to 5.97×10^{-4} M at pH 7.40 and 37 °C. As reported previously (*14*, *16*), the chiral inversion of thalidomide was found to be catalyzed by HSA. The observed pseudo-first-order rate constants of chiral inversion were linearly dependent on HSA concentration (Figure 3). Furthermore, the comparison of the rates of chiral inversion of (*R*)- and (*S*)-thalidomide, $k_{(R\text{-to-}S)}$ and $k_{(S\text{-to-}R)}$, showed a slight HSA concentration-dependent stereoselectivity (Figure 3). At low albumin concentrations of up to 1.5×10^{-4} M, no significant difference between the rates of inversion of the two enantiomers was observed. At a physiological concentration of fatty acid-free HSA (5.97×10^{-4} M) however, the inversion from (*S*)- to (*R*)-thalidomide was about 1.4 times faster than that from the *R*- to the *S*-enantiomer.

The hydrolysis of thalidomide was not influenced much by HSA, except for a marginal inhibition (Figure 3). There was no significant difference in the rates of hydrolysis between the two enantiomers at all HSA concentrations investigated.

Influence of pH on the Chiral Inversion and Hydrolysis of Thalidomide. The chiral inversion and

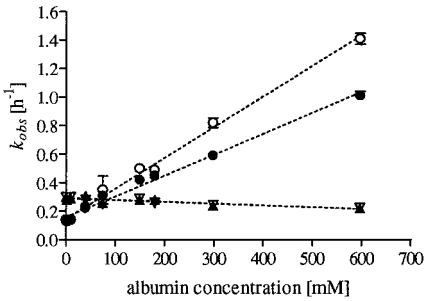

Figure 3. Influence of the concentration of fatty acid-free HSA (Sigma A-1887) on the chiral inversion and hydrolysis of thalidomide (HSA solutions in 0.1 M phosphate buffer at pH 7.40, an ionic strength of 0.3, and 37 °C). k_{obs} is the observed pseudo-first-order rate constant of chiral inversion or hydrolysis: (○) inversion from the *S*- to *R*-configuration, (●) inversion from the *R*- to *S*-configuration, (▽) hydrolysis of (*S*)-thalidomide, and (▲) hydrolysis of (*R*)-thalidomide. Values are means ± standard deviation of triplicate determinations. Some standard deviations were smaller than the symbols.

hydrolysis of thalidomide enantiomers in phosphate buffers were found to be pH-dependent. The values for the observed pseudo-first-order rate constants of enantiomerization k_{enant} and of hydrolysis k_{hyd}, as obtained in a series of 0.1 M phosphate buffers over a pH range of 2−8 at 37 °C, are compiled in Table 1. Hydrolysis of thalidomide at pH 11 was too fast to be followed. Thus, no rates of chiral inversion or hydrolysis could be determined at this pH. Both rates are about zero at low pH and were found to increase with pH, indicating that chiral inversion and hydrolysis of thalidomide are base-catalyzed processes. The rates of hydrolysis were found to be faster than those of enantiomerization at all pH values investigated. As expected, no stereoselectivity of chiral inversion or hydrolysis was found in 0.1 M phosphate buffers over the pH range investigated. The reaction rates of (*R*)- and (*S*)-thalidomide are the same within limits of error (Table 1).

Influence of Phosphate Concentration on the Chiral Inversion and Hydrolysis of Thalidomide. Apparent pseudo-first-order rate constants of enantiomerization k_{enant} and hydrolysis k_{hyd} of thalidomide, measured at a constant pH (7.4), ionic strength (0.8), and temperature (37 °C), were linearly dependent upon phosphate concentration (Figure 4). Equations 5 and 6 describe this linear dependence for chiral inversion and hydrolysis, respectively:

$$k_{enant} = 0.24(\pm 0.017)C_{phosphate} + 0.081(\pm 0.003)$$
$$n = 4,\ r^2 = 0.990,\ s = 0.003,\ F = 201 \qquad (5)$$

$$k_{hyd} = 0.55(\pm 0.073)C_{phosphate} + 0.23(\pm 0.013)$$
$$n = 4,\ r^2 = 0.965,\ s = 0.014,\ F = 56 \qquad (6)$$

where n is the number of pH values investigated, r^2 the squared correlation coefficient, s the standard deviation of the residuals, and F the Fisher test for significance of the equation (standard deviations are given in parentheses).

Again, the rates of hydrolysis were found to be faster than those of enantiomerization at all phosphate concentrations investigated, and no stereoselectivity was detected. The rates of reaction of (*R*)- and (*S*)-thalidomide were the same within limits of error.

Table 1. pH Dependence of the Observed Pseudo-First-Order Rate Constants[a] of Enantiomerization and Hydrolysis of Thalidomide (0.1 M phosphate buffer with an ionic strength of 0.3 at 37 °C)

	k_{enant} (h^{-1})		k_{hyd} (h^{-1})	
pH	*R*	*S*	*R*	*S*
2.03	<0.001	<0.001	<0.01	<0.01
3.58	<0.001	<0.001	<0.01	<0.01
6.06	0.010 ± 0.0004	0.010 ± 0.0011	0.04 ± 0.024	0.04 ± 0.024
7.44	0.121 ± 0.0035	0.112 ± 0.0041	0.32 ± 0.013	0.33 ± 0.010
8.03	0.34 ± 0.031	0.32 ± 0.067	1.25 ± 0.052	1.27 ± 0.019

[a] Rate constants of enantiomerization k_{enant} were calculated from experimentally determined rate constants of racemization k_{rac} (=2k_{enant}) (*29*). Values are means ± standard deviation of triplicate determinations.

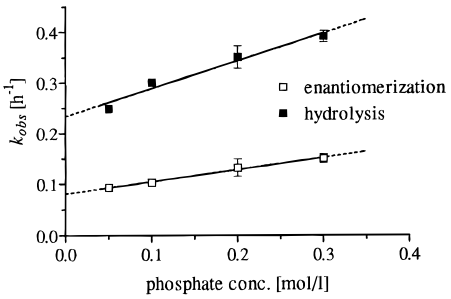

Figure 4. Influence of phosphate concentration on thalidomide enantiomerization and hydrolysis (pH 7.4, ionic strength of 0.8, 37 °C). k_{obs} is the observed pseudo-first-order rate constant of enantiomerization or hydrolysis. Values are means ± standard deviation of triplicate determinations. Some standard deviations were smaller than the symbols.

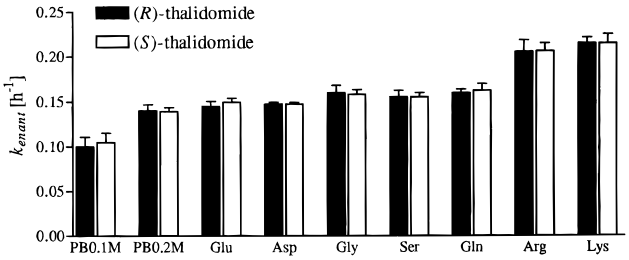

Figure 5. Influence of various amino acids on the enantiomerization of thalidomide (solutions of amino acids in 0.1 M phosphate buffer at pH 7.4, an ionic strength of 0.9, and 37 °C): (PB0.1M) 0.1 M phosphate buffer at pH 7.4 and an ionic strength of 0.9 and (PB0.2M) 0.2 M phosphate buffer at pH 7.4 and an ionic strength of 0.9. Values are means ± standard deviation of triplicate determinations.

Influence of Various Amino Acids on the Chiral Inversion and Hydrolysis of Thalidomide. The apparent pseudo-first-order rate constants of enantiomerization and hydrolysis of thalidomide were determined in 0.1 M solutions of various acidic, neutral, and basic amino acids. The temperature, pH, and ionic strength were kept constant. Acidic (Glu and Asp) and neutral (Ser, Gln, and Gly) amino acids (i.e., with only one basic group) catalyzed the chiral inversion of thalidomide to the same extent as a phosphate buffer with the same molarity. In contrast, basic amino acids (Arg and Lys) showed a higher catalytic potency (Figure 5). The hydrolysis of thalidomide was catalyzed to the same extent by all amino acids investigated or by a phosphate buffer with the same molarity (data not shown). No stereoselectivity was detected in any of these experiments.

Configurational Stability and Hydrolysis of the Three Teratogenic Thalidomide Metabolites As

Examined by ^{1}H NMR. The configurational stability and hydrolysis of the three teratogenic metabolites of thalidomide **2**, **3**, and **5** (Figure 1) were monitored by ^{1}H NMR. In a 0.3 M phosphate buffer at pD 7.4 and 37 °C, all three metabolites underwent hydrolysis. However, no deuteration was observed for any of the three metabolites after up to 7 days of incubation. There was no observable decrease of the sum of the integrals of the signal of the proton coupled to the chiral center of the teratogenic metabolites (**2**, **3**, and **5**) and that of the proton coupled to the chiral center of the hydrolyzed metabolites (i.e., **6**−**8**). The ratios of (sum of the integrals of the protons of the chiral centers)/(integral of the signal of the four aliphatic reference protons in the α- and *β*-positions with respect to the chiral center) at time zero and after 7 days were identical. Hence, the configuration of the three teratogenic thalidomide metabolites **2**, **3**, and **5** (Figure 2) in a phosphate buffer (0.3 M, pD 7.4) at 37 °C was found to be completely stable for at least 7 days. The half-lives of hydrolysis under these conditions were estimated to be 6 h for 4-phthalimidoglutaramic acid (**2**), 49 h for 2-phthalimidoglutaramic acid (**3**), and 47 h for 2-phthalimidoglutaric acid (**5**). For the same compounds at pH 7.4 and 37 °C, Schumacher et al. (*19*) reported hydrolysis half-lives of 5, 48, and 43 h, respectively.

Discussion

Mechanism of Chiral Inversion. The chiral HPLC assay described in this study was shown to be suitable for the simultaneous determination of the chiral inversion and hydrolysis of thalidomide in vitro. Good accuracy and precision were shown at all *R*/*S* and thalidomide/anthracene concentration ratios. The simultaneous determination of the chiral inversion and hydrolysis of thalidomide using only one chiral HPLC column is novel.

As expected (*14*, *16*), human serum albumin catalyzed the chiral inversion of thalidomide. The observed pseudo-first-order rate constants of chiral inversion increased with increasing HSA concentration. To obtain further insights into the mechanism of chiral inversion of thalidomide and its catalysis by HSA, the influence of pH, phosphate concentration, and various acidic, neutral, and basic amino acids was investigated. Our results showed that the chiral inversion of thalidomide was pH-dependent. At acidic pH, the rate of chiral inversion was about zero, and it increased with increasing pH. This suggests that the chiral inversion of thalidomide is a base-catalyzed process. It was also shown that at pH 7.4 the rate of inversion was linearly dependent on phosphate concentration. Thus, chiral inversion is deduced to be subject to general base catalysis. Further information about the catalytic species involved in phosphate buffers at pH 7.4 can be obtained from eq 5, which represents the linear dependence of chiral inversion upon phosphate concentration. The intercept at zero buffer concentration is 0.081(±0.003), thus different from zero, and it can be presumed that at pH 7.4 uncatalyzed and specific base-catalyzed reactions take place along with general base-catalyzed reactions. Hence, the chiral inversion of thalidomide in phosphate buffers at pH 7.4 is catalyzed by the basic component of the phosphate buffer (HPO_4^{2-}) as well as by hydroxyl ions and water molecules.

The influence of various acidic, neutral, and basic amino acids on the chiral inversion of thalidomide showed that the basic amino acids Arg and Lys (which possess two basic groups) have a greater catalytic potency than neutral and acidic amino acids that possess only one basic group. This also implies a general base catalysis in the chiral inversion of thalidomide. Albumin has many reactive ϵ-amino groups (*37*), and almost all of its ligand binding sites involve lysine and/or arginine residues (*37*, *38*). For example, one of the most prominent of all of these residues is Lys-199, which is involved in the binding of acetylsalicylic acid (ASA). Previous studies (*14*, *16*) have shown that various albumin ligands such as long- and medium-chain fatty acids as well as ASA were able to partly inhibit the ability of HSA to catalyze the chiral inversion of thalidomide. However, these ligands do not share the same binding site on albumin. ASA binds to Sudlow's site I; medium-chain fatty acids bind to Sudlow's site II, and long-chain fatty acids bind to about six sites (*37*, *38*). Yet these binding sites all involve reactive lysine and arginine residues (*37*, *38*). Thus, the binding of a ligand could block these basic amino acid residues, rendering them nonavailable for catalyzing the chiral inversion of thalidomide. On the basis of these arguments and on the basis of the findings of this study that the chiral inversion of thalidomide is subject to general base catalysis, it is suggested that the ability of albumin to catalyze the chiral inversion of thalidomide is due to its Arg and Lys residues. No single catalytic site on the albumin molecule is thought to exist. This suggestion is also consistent with the fact that the chiral inversion of thalidomide in vivo, in plasma, and in blood was found to be slower than in HSA solutions with physiological concentrations (*15*, *16*). Endogenous albumin ligands such as fatty acids and bilirubin may block the catalytic basic amino acid residues of HSA and as a result reduce the extent of catalysis of the chiral inversion.

Furthermore, the rates of chiral inversion of (*R*)- and (*S*)-thalidomide showed a slight HSA concentration-dependent stereoselectivity. At low albumin concentrations, no significant difference between the rates of inversion of the two enantiomers could be observed, whereas at physiological concentrations, the inversion from (*S*)- to (*R*)-thalidomide was about 1.4 times faster than that from the *R*- to the *S*-enantiomer. This may be due to the fact that albumin is subject to concentration-dependent polymerization caused by various factors such as heating, freeze-drying, etc. (*39*, *40*). For the commercial fatty acid-free HSA used in this study, which is freeze-dried, it is known that at low albumin concentrations no polymerization occurs whereas at a physiological concentrations up to 50% of the HSA can be polymerized (*40*). This polymerization might lead to conformational changes in the HSA molecule, inducing stereoselectivity by an unknown mechanism. This hypothesis is in agreement with the finding that in freshly drawn blood no significant difference could be observed between the rates of chiral inversion of the enantiomers. No albumin polymerization is believed to exist in untreated human blood (*38*). In vivo in humans, it was even found that (*R*)-thalidomide inverses its configuration faster than (*S*)-thalidomide (*15*). Hence, the stereoselectivity of the chiral inversion of thalidomide is dependent on the medium, and it would be misleading to draw conclusions about in vivo stereoselectivity from in vitro studies.

In summary, the chiral inversion of thalidomide is suggested to occur by electrophilic substitution with protons as incoming and leaving groups. Specific and

general base catalysis accelerates the reaction by facilitating the abstraction of the proton bound to the chiral center.

Mechanism of Hydrolysis. The hydrolysis of thalidomide, like its chiral inversion, was found to be base-catalyzed. From eq 6, which represents the linear dependence of the rate of hydrolysis on phosphate concentration, it can be deduced that at pH 7.4 the hydrolysis of thalidomide, like its chiral inversion, is catalyzed by the basic component of the phosphate buffer as well as by hydroxyl ions and water molecules. We showed that the hydrolysis of thalidomide is catalyzed by amino acids. However, in contrast to chiral inversion, there was no difference between the catalytic potencies of acidic, neutral, and basic amino acids, and albumin did not catalyze the hydrolysis of thalidomide. This may be explained by a different reaction mechanism of chiral inversion and hydrolysis. Whereas chiral inversion is suggested to occur by electrophilic substitution, the reaction mechanism of hydrolysis is thought to be a nucleophilic substitution involving specific and general base catalysis as well as nucleophilic catalysis. Proton transfer (e.g., base catalysis) is rather insensitive to steric factors since the proton is so small, whereas nucleophilic attack is very sensitive to the size of both the reactant and catalyst (*41*). With regard to its size, it is likely that albumin is efficient as a base catalyst but not as a nucleophilic catalyst. This might explain why albumin catalyzes chiral inversion, but not the hydrolysis of thalidomide. Steric hindrance might also account for the fact that basic amino acids were indeed more effective than neutral and acidic ones in catalyzing chiral inversion but not hydrolysis. In theory, hydrolysis might also occur via an E1cb mechanism and a ketene intermediate stemming from the same carbanion involved in chiral inversion and deuterium exchange. But the absence of catalysis of hydrolysis by albumin suggests that this mechanism is rather improbable.

Chiral Stability of Teratogenic Metabolites. The configuration of the three teratogenic metabolites of thalidomide in phosphate buffer at pH 7.4 was found to be stable for at least 7 days. ^{1}H NMR has previously been shown to be a suitable tool for assaying racemization of chiral centers of the type R″R′RCH (*30*−*33*, *36*). Here, the configurational stability of the teratogenic metabolites of thalidomide is discussed in connection with a possible enantioselectivity on the teratogenicity of thalidomide. Given the stable configuration of the three teratogenic metabolites, inversion of the chiral center must stop with the hydrolysis of thalidomide. Thus, after administration of (*S*)-thalidomide, the concentration of teratogenic metabolites with the *S*-configuration is postulated to be higher than after application of (*R*)-thalidomide. If it is assumed that the teratogenic potency of the metabolites with the *S*-configuration is markedly superior to that of the metabolites with the *R*-configuration, as verified for 2-phthalimidoglutaric acid (**5**) (*23*, *24*), it might in fact be conceivable that (*R*)-thalidomide could cause less teratogenic effects. From a more practical point of view, the high configurational stability of the three teratogenic metabolites of thalidomide may be useful in assaying the biological activities of the single enantiomers.

In conclusion, the chiral inversion of thalidomide has been shown to be subject to specific and general base catalysis. It is suggested that the ability of HSA to catalyze the chiral inversion of thalidomide is due to the basic functional groups of the amino acids arginine and lysine in general and not to a single catalytic site. Further, the configurational stability of the three teratogenic metabolites allows us to speculate that (*R*)-thalidomide might be less teratogenic than the *S*-enantiomer.

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Supporting Information Available: Detailed calculations for the estimation of the rate constants of chiral inversion and hydrolysis and discussion of the use of ^{1}H NMR (^{1}H/^{2}H substitution) as a tool for assaying the racemization of chiral centers of the type R″R′RCH (6 pages). Ordering information is given on any current masthead page.

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