Absorption of Thalidomide in the Rat. (27252)

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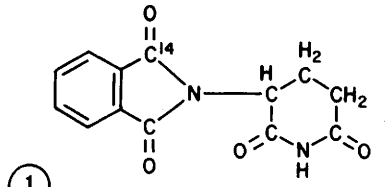
Thalidomide [N-phthaloyl glutamimide, Kevadon®, Contergan®] has undergone extensive clinical trials as a sedative and hypnotic(1,2). It has been speculated(3,4,5) that its lack of toxicity is associated with limited absorption. These speculations are largely based upon the insolubility of thalidomide in water. This paper reports the excretion pattern of orally administered C^{14} labelled thalidomide and its concentration in blood and brain as a function of time.

Methods. Thalidomide was labelled with C^{14} in one of the carbonyl groups of the phthaloyl moiety (Fig. 1). The specific activity was 4.7 μc/mg. This was supplied by Orlando Research, Inc. Samples were counted in a Nuclear-Chicago D-47 gas flow counter with a Micromil window. All samples were corrected for self-absorption, and counting was continued long enough to reduce statistical error below 5%. Experimental animals were male Sprague-Dawley rats weighing approximately 250 g for the excretion study and 350 g for the blood and brain level study. All rats received Purina chow and water *ad lib.* Urine was plated directly and counted for radioactivity; feces were homogenized in dilute NaOH, plated and counted. Samples of blood or brain homogenates were shaken with an equal volume of dioxane for 15 minutes in a mechanical shaker, and the resulting suspension centrifuged. The supernatant was collected and extracted 3 times with a volume of chloroform equal to twice the volume of the original sample. The combined chloroform extracts were concentrated for chromatography. This procedure recovered 98% of thalidomide radioactivity added to brain or plasma, and 80-90% of that added to whole blood.

Results. Blood and brain levels following oral thalidomide-C^{14}. Radioactive thalidomide was administered orally at a dose of 10 mg/kg to groups of 4 rats. These were sacrificed at selected intervals; blood and brains were collected for analysis. Blood samples from individual rats were plated directly and counted, after which a pooled sample from each group was extracted and chromatographed in order to identify the radioactive material present. Individual brains were counted by homogenizing in dioxane-ethanol (1:1) and taking an aliquot for plating. The remaining homogenates were extracted and chromatographed.



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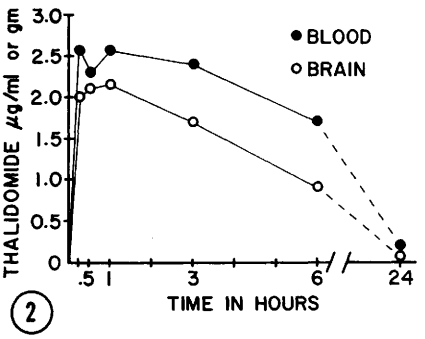

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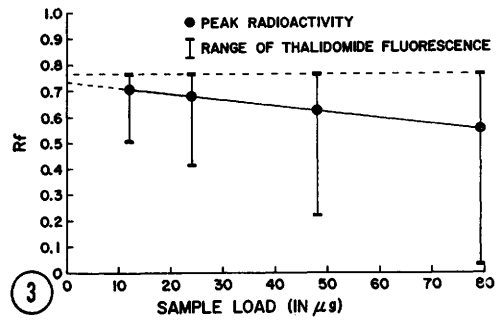

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FIG. 1. Structural formula for thalidomide and position of C^{14} label.

FIG. 2. Concentrations in blood or brain after 10 mg/kg of thalidomide in the rat.

FIG. 3. Effect of various amounts of thalidomide-C^{14} on its R_f values and distribution in the n-butanol-NH_4OH system.

TABLE I. Concentration of Thalidomide-C^{14} in Blood and Brain at Various Intervals Following Oral Administration of 10 mg/kg.

Interval	Whole blood* (μg/cc) Avg	Range†	Extracted μg/cc	%
15 min.	2.58	2.0 -3.8	2.3	88
30 "	2.30	2.2 -2.4	2.1	90
1 hr	2.58	2.5 -2.6	2.1	80
3 "	2.39	2.2 -2.5	2.0	82
6 "	1.62	1.3 -1.8	1.3	73
24 "	.21	.20- .23	.04	16

Interval	Brain* (μg/g) Avg	Range†	Extracted ‡ μg/g	%
15 min.	2.0	1.9 -2.1	1.5	76
30 "	2.1	1.8 -3.0	1.2	57
1 hr	2.2	1.9 -2.4	1.7	78
3 "	1.7	1.5 -1.9	.85	50
6 "	.9	.7 -1.0	.68	76
24 "	.05	.05- .06	.01	20

* Calculated from radioactivity. All radioactive material was assumed to be thalidomide.

† Range of values from 4 rats.

‡ Amount actually recovered; figures might be higher since some of all the brain samples was lost.

Fig. 2 shows the average levels of thalidomide in blood and brain at different time intervals, based on the assumption that all the radioactivity represented intact thalidomide. The validity of this assumption is considered below. Up to 6 hours after drug administration the radioactivity in blood and brain was extractable to the same extent as thalidomide (Table I). Since the primary metabolic degradation would probably involve opening of the glutamimide ring, the resulting products, phthaloyl-glutamine or phthaloyl-glutamic acid, would be ionized at physiological pH and stay therefore in the water phase. In contrast, 24 hours after the drug administration 20% or less of the radioactivity was extractable with chloroform, indicating that most of the radioactive material was not thalidomide but a more polar metabolic product. Since the total amount of radioactive material present at this time in blood or brain was very small, no further identification was made.

Identification of radioactive material in blood and brain. For chromatographic separation and identification of thalidomide, 2 solvent systems were used. The first was that of Beckmann and Kampf(6), consisting of dimethylformamide (DMF), methanol, and water (25:70:5), and the second was n-butanol saturated with 0.2% ammonium hydroxide. Thalidomide and several of its potential metabolites were chromatographed, with the results given in Table II. The DMF-methanol-water system had very limited usefulness in separation of thalidomide from the ring-opened compounds that are hydrolytic products. However, in this system thalidomide could be distinguished from these other compounds, except phthalimide, by its fluorescence under ultraviolet light. The breakdown products of thalidomide exhibited quenching of the background fluorescence of the paper, and thus appeared as dark spots under ultraviolet illumination. The n-butanol-NH_4OH system separated thalidomide from the ring-opened compounds. Unfortunately, in this system, tailing occurred with samples containing more than 10 μg of material per application. Fig. 3 shows that when increasing amounts of thalidomide-C^{14} were applied to Whatman No. 1 paper, there was an increase in tailing and a decrease in R_f of *peak* radioactivity. Nevertheless, the R_f value of thalidomide, when measured at its most advanced location, as seen under ultraviolet light, was constant no matter how much was added. Tailing, due to overloading, has been reported for other chromatographic systems. Because of the presence of large amounts of lipoidal material in the brain, the specific activity of the soluble residue was much lower than that of blood. The amount of brain extract required to provide enough radioactivity for location on the chromatogram was therefore much larger than for blood extract. This excess of material resulted also in retardation of the radioactivity peak, but the fluorescent leading edge of thalidomide in the chromatograms of brain extracts was again the same as that for the pure thalidomide (Table II). Thus, tailing was not restricted to overloading alone, but could be caused also by the presence of other material applied to the paper. For these reasons, the R_f values reported in Table II were measured to the leading edge of each solute, as seen under ultraviolet light.

TABLE II. R_f Values* of Thalidomide, Potential Metabolites, Tissue Extracts and Urine from Rats Receiving Thalidomide-C^{14} Orally.

Material	Solvents: DMF : MeOH : H_2O 25 : 70 : 5 R_f	Solvents: n-butanol sat. with .2% NH_4OH R_f	Spot appearance in UV light
Crystalline thalidomide	.8	.8	Fluorescent
Phthaloyl DL-glutamine	.8	.1	Dark
Phthaloyl DL-glutamic acid	.8	.04	"
NaOH + phthaloyl glutamic acid	.2	—	"
KOH + phthaloyl glutamic acid	.06	—	"
Phthalic acid	.86	.2	"
Phthalimide	.91	.9	Fluorescent
Extract from rat plasma	.8	.8	"
" " " brain	—	.8	"

* R_f values were measured at solute front.

R_f values for peak radioactivity in the n-butanol-NH_4OH system were 0.62 for blood extracts and 0.45 for brain extracts in the thalidomide absorption study.

Further identification of the radioactive material in the blood and brain extracts was carried out by recrystallization of this material in the presence of carrier thalidomide. The extracts from each tissue at the various time intervals were combined to increase the amount of radioactivity. Then 200 mg of non-radioactive, crystalline thalidomide was added to each pooled extract and the extracts recrystallized from dioxane, filtered, washed with ether, plated and counted. Three additional recrystallizations and washings, first with ether and then ethanol, were performed following which the thalidomide was again plated, weighed, and counted. The results indicated that specific radioactivity was maintained constant through 3 recrystallizations and washings, and that 80 and 75% of the radioactivity in blood and brain extracts, respectively, recrystallized as thalidomide.

Excretion of thalidomide-C^{14}. Fasted rats were given aqueous suspensions of labelled thalidomide by stomach tube and urine and feces were collected separately from individual animals during the time intervals indicated in Table III. Three groups of 4 rats received respectively 10, 100, and 1000 mg/kg. All doses contained approximately 3 μc of radioactivity and the differences in dose were made up by dilution with non-radioactive drug.

At the lowest dose approximately 40% of the administered radioactivity was excreted in the urine, with more than half of it appearing within 6 hours (Table III). With 100 mg/kg, the peak of urinary excretion occurred somewhat later, but the same fraction of the dose was excreted in the urine. At 1000 mg/kg, the peak urinary excretion still occurred between 6 and 24 hours, and the fraction of the dose thus excreted was nearly 30%. Evidently, urinary excretion is proportional to dose over a wide dose range. Radioactive material in the feces was excreted largely between 6 and 24 hours at all dose levels.

To determine whether the material excreted in the urine was thalidomide, it was chromatographed in the DMF-methanol-water system. No radioactivity was found at the R_f position corresponding to thalidomide. Radioactive spots were found at R_f 0.2 and 0.07, corresponding to the sodium and potassium salts of phthaloyl glutamic acid (Table II). When radioactive thalidomide is dissolved in NaOH or KOH and chromatographed in the DMF-methanol-water system, R_f values for radioactive peaks are .2 and .07 respectively. In feces, half the radioactivity was extractable with the dioxane-chloroform extraction procedure. When chromatographed in the n-butanol system, the solute front under U.V. light had an R_f 0.89; radioactive peaks were found at R_f 0.65, and R_f 0.27. The first corresponds to the R_f of thalido-

TABLE III. Percentages of Administered Radioactivity Appearing in Urine and Feces at Various Intervals after Oral Administration of Thalidomide-C^{14}.

Excretion	Interval (hr)	% injected dose 10 mg/kg	100 mg/kg	1000 mg/kg
Urine	0- 6	24.9 (22.0- 26.3)*	15.4 (12.5- 21.5)	8.3 (5.1- 10.6)
"	6-24	14.7 (11.4- 17.8)	22.8 (18.1- 27.9)	17.3 (15.9- 18.6)
"	24-48	1.2 (.9- 1.8)	1.2 (.9- 1.7)	3.2 (1.7- 4.6)
Total urinary excretion		40.8 (34.3- 45.9)	39.4 (31.5- 51.1)	28.8 (22.7- 33.8)
Feces	0- 6	2.3 (.3- 4.4)	1.4 (.0- 4.3)	2.4 (.1- 8.0)
"	6-24	49.3 (43.0- 58.6)	46.5 (40.6- 50.8)	54.5 (50.5- 59.0)
"	24-48	3.0 (2.0- 3.7)	2.8 (2.0- 4.0)	8.9 (3.4- 14.0)
Total fecal excretion		54.6 (45.3- 66.7)	50.7 (42.6- 59.1)	65.8 (54.0- 81.0)
Total recovery		95.4 (79.6-112.6)	90.1 (74.1-110.2)	94.6 (76.7-114.8)

* Range of values from 4 rats in parentheses.

mide. The second did not correspond closely enough to any of the values for known compounds to provide a tentative identification. The remaining half of the fecal radioactivity was extractable with water. This radioactivity did not move from the origin when chromatographed in the n-butanol system. This does not serve to identify the urinary and fecal materials completely, but it is certain that thalidomide itself was not excreted in measurable amounts in the urine.

Discussion. Following oral administration of radioactive thalidomide to rats, appreciable levels of radioactive material were recovered from blood and brain (Fig. 2). Most, if not all, of this material was intact thalidomide as judged by the following: Up to 6 hours after drug administration the radioactivity was extractable to the same extent as thalidomide from these tissues. The extracted radioactivity chromatographed with the same R_f characteristics as thalidomide. It also fluoresced under ultraviolet light (as does thalidomide). R_f values and fluorescence distinguish the material from expected hydrolytic products (Table II). Addition of carrier thalidomide to this material, followed by multiple recrystallizations from dioxane and washings with ether and ethanol, showed that following an initial loss of 20% (blood) or 25% (brain), the radioactive material was indistinguishable from thalidomide by maintaining constant specific activity through 3 subsequent recrystallizations and washings.

Peak blood levels occurred 15 minutes after oral administration of thalidomide, indicating rapid absorption from the gastro-intestinal tract. Since peak concentrations in brain were reached in 30 to 60 minutes, entrance from the blood into brain also occurs rapidly. At 6 hours, the concentration in brain had fallen to 41% of the peak value, and in blood to 63%. At 24 hours, radioactive concentration in brain and blood had decreased to 2% and 8% of their respective peak values. At this time about 20% of the radioactive material behaved as thalidomide. Evidently, the remainder represented degradation products of thalidomide.

The excretion data indicate that at least 40% of the labelled material is absorbed over a wide dose range (10-100 mg/kg). With 1000 mg/kg, this average decreased to 30%. Urinary and fecal excretion of the label occurred rapidly, and the amount remaining in the animal 48 hours after administration averaged 10% of the dose, or less. Since with the largest dose (1000 mg/kg) one-fourth appeared in the urine within 24 hours, the excretory capacity of the kidney for the absorbed material was at least that high during this time. The radioactive material in the feces could represent unabsorbed thalidomide, but may also include some which was absorbed and later secreted into the gastro-intestinal tract.

Summary. Thalidomide is rapidly absorbed from the gastro-intestinal tract. The peak blood level appeared in 15 minutes and peak brain level occurred between 30 minutes and 1 hour. Identification of the radioactive label in blood and brain indicates that

this is thalidomide and not a metabolite of thalidomide. The radioactive material in the urine is not thalidomide. Over a wide range (10-1000 mg/kg) an average of 30 to 40% of the radioactive label appeared in the urine in 48 hours. During this same period, most of the remaining radioactive label appeared in the feces.

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Action of Antibodies on Metabolism of Normal and Tumor Cells *in vitro*. (27253)

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Many workers have studied the cytotoxic action of antisera on normal and tumor cells *in vitro*. Some investigators used phase contrast microscopy or electron microscopy to study the morphological changes produced by the action of antisera(1-6), and changes in permeability of cytoplasmic membranes to dyes(7,8). Other experiments compared capacity of growth, *in vitro* or *in vivo*, after the action of antiserum(2,3,8,9,10).

In general these investigations showed that after a more or less prolonged incubation period in presence of antibodies, there were changes of the cytoplasmic membrane; swelling with formation of cytoplasmic blisters, with following swelling and fragmentation of the mitochondria; heaping of cytoplasmic granules around the nucleus, vacuolization and enhanced cell fragility, and, in the end, loss of cytoplasmic material. Ellem(11) showed an increased permeability of the cytoplasmic membrane to inorganic and organic acid-soluble phosphorus, which goes from the cell into the culture medium. Green *et al.* (12) observed that the incubation with antiserum elicits a decrease of RNA and cytoplasmic proteins, free amino acids, ribonucleotides and potassium, while DNA content remains unchanged. These changes are more evident in the presence of complement.

The action of antibodies on cell metabolism has been investigated less extensively. Flax (3) observed that under the action of immune gamma globulins, in presence of complement, Ehrlich ascites cells cannot utilize exogenous glucose by the oxidative pathway; on the contrary, they utilize succinate. Using the same cell type, Sugai and Aizawa(5) and Bickis *et al.*(6), showed that antiserum causes inhibition of the respiration in absence of substrates under the same conditions, anaerobic glycolysis is also inhibited, while the respiration with succinate is enhanced(6).

Recently it has been shown that normal human sera cause inhibition of aerobic and anaerobic glycolysis of sarcoma 37 ascites, and of anaerobic glycolysis of the Ehrlich and Krebs ascites carcinomas(8), as well as respiration of the latter 2 tumors(13). This action is complement-linked and may be due to presence of natural antibodies.

In our experiments we analyzed the behavior of respiration and aerobic glycolysis as a consequence of the action of specific antibodies, considering 2 cell lines; cells of normal kidney and of epidermoid carcinoma, cultured *in vitro*, and cells from the rat liver and Walker rat carcinosarcoma 256.

Methods and material. Normal kidney cells (PF) and epidermoid carcinoma cells of the floor of the mouth (KB) were cultured *in vitro* in Hanks' medium(14) supplemented