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THE METABOLISM OF THALIDOMIDE: SOME BIOLOGICAL EFFECTS OF THALIDOMIDE AND ITS METABOLITES

BY

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In the preceding paper (Schumacher, Smith & Williams, 1965) it was shown that thalidomide was converted in the body into a number of compounds, several of which are derivatives of the amino acids, glutamic acid and glutamine. Since thalidomide is racemic, some of its breakdown products will, therefore, contain amino acid residues of the unnatural D-series. Any of the biological effects (embryotoxic, neurotoxic and sedative) of thalidomide could be due to the drug or its metabolites interfering with the normal metabolism of L-glutamine and L-glutamic acid. These amino acids are prominently involved in the metabolic activity of the central and peripheral nervous system (Tower, 1960), although their role in foetal development is not clear. There are indications, however, that they may be involved in morphogenesis (Deuchar, 1962).

In this paper the effects of thalidomide and its hydrolysis products on three enzymes concerned with glutamate metabolism (namely glutamine synthetase, glutamate decarboxylase and glutamate dehydrogenase), on the embryos of pregnant rabbits and on the hexobarbitone-induced sleeping time in rats, will be described.

METHODS

Glutamine synthetase. An acetone-dried powder of rat brain was prepared. Brains from freshly killed adult female rats were rapidly removed, cooled on ice, weighed and homogenized in ten volumes of ice-cold acetone. The resulting suspension was centrifuged at 4,000 revs/min for 10 min and the acetone was poured off. The solid residue was resuspended in a further ten volumes of ice-cold acetone and centrifuged as before. The acetone layer was again decanted and the solid residue was dried at 4° C *in vacuo* over silica gel and paraffin wax. To prepare an extract containing glutamine synthetase activity, the acetone-dried powder (0.5 g) was homogenized for 2 min in 10 ml. 0.1 M-tris-potassium chloride buffer (*p*H 7.3) at 0° C using a Waring blender. The mixture was centrifuged at 4,000 revs/min for 5 min and the supernatant fluid was used as a source of glutamine synthetase activity, which was estimated by the method of Rudnick, Mele & Waelsch (1955). A typical incubation mixture contained the following: sodium L-glutamate (100 μmoles), adenosine triphosphate (10 μmoles), magnesium chloride (40 μmoles), 2-amino-2-(hydroxymethyl)propane-1,3-diol hydrochloride (tris) (100 μmoles), potassium cyanide (80 μmoles), hydroxylamine (40 μmoles) and the enzyme solution (0.3 ml.) in a final volume of 2 ml. Substances to be tested for inhibitory activity were dissolved in the tris buffer. The low solubility of thalidomide precluded it from being tested at the same concentration as those used for other compounds. Hydroxylamine was freshly prepared before use by neutralizing hydroxylamine hydrochloride with 2 N-sodium hydroxide. The preparations were incubated at 37° C for 20 min and then the reaction was stopped by adding 1.5 ml. of a solution containing equal volumes of 5% (w/v) ferric chloride (in 0.1 N-hydrochloric acid), 2.5 N-hydrochloric acid and 15% (w/v) trichloracetic acid. The reaction mixtures were clarified by centrifuging and the optical

density of the supernatant fluid was measured at 500 mμ in a 1-cm cell (Unicam Spectrophotometer SP 500). The amount of γ-glutamylhydroxamate formed enzymically was then found by the use of a standard curve prepared from known amounts of γ-glutamylhydroxamic acid. Results were expressed as μmoles γ-glutamylhydroxamate formed per g of acetone powder per hr.

L-Glutamic acid decarboxylase. Fresh rat brains were rinsed in ice-cold phosphate buffer (0.05 M, *p*H 6) and weighed, and a 25% homogenate in phosphate buffer was prepared using a Potter homogenizer. Assays were carried out manometrically using a standard Warburg apparatus at 37° C. The main compartment of the vessel contained 1 ml. of enzyme preparation (equivalent to 220 to 270 mg of wet brain), pyridoxal phosphate (400 μg in 0.2 ml.) and 1.1 ml. of phosphate buffer (0.05 M, *p*H 6.2) containing, where appropriate, the substance under test. The side arm contained 0.5 ml. of aqueous monosodium L-glutamate (75, 125 or 250 μmoles). The inhibitory action of each compound was tested at each of these concentrations of glutamate. The final *p*H of the incubation mixture was 6.2 to 6.3.

The manometers were flushed with oxygen-free nitrogen for 7 min and equilibrated at 37° C for 10 min. At zero time the substrate was tipped in, and manometer readings of the carbon dioxide output were made at 10-min intervals. After 1 hr, 2 N-sulphuric acid (0.2 ml.) was added and a final reading was taken 10 min later. This final figure was used in the calculation of the enzyme activity while the readings at 10-min intervals were used to check that the carbon dioxide output maintained a linear relationship with time.

Glutamate dehydrogenase. The acetone-dried powder of rat brain was used as a source of enzyme activity. The powder (1 g) was homogenized at 0° C with 10 ml. of 0.1 M-phosphate buffer (*p*H 7.6) for 10 min in a Waring blender. The homogenate was centrifuged at 4,000 revs/min for 10 min and the supernatant fluid was used for incubation. The protein content of the supernatant fluid was determined spectrophotometrically by measuring the ratio of the extinctions at 260 and 280 mμ (Kalckar, 1947). A typical incubate (3 ml.), contained in a 1-cm silica cuvette, consisted of the following: 0.1 M-phosphate buffer, *p*H 7.8 (1 ml.); sodium L-glutamate (30 μmoles); NAD (nicotinamide-adenine dinucleotide; 0.75 μmoles); enzyme preparation (0.25 ml., approximately equivalent to 5 mg of protein). Compounds to be tested as inhibitors were added to incubate as a solution (0.5 ml.) in 0.15 M-phosphate buffer, *p*H 7.8. The reaction was initiated by addition of the NAD and the course of the reaction followed at room temperature (20° C) by measuring the changes in optical density at 340 mμ at 30-sec intervals, over a period of 5 min. The reaction rate was linear only over the first 2 min due to product inhibition (Caughey, Smiley & Hellerman, 1957). The activity of the enzyme was calculated from the rate of reaction over the initial 2-min period and results were expressed as μmoles of $NADH_2$ formed per min per g of protein.

Embryotoxicity of thalidomide and related compounds in rabbits. The embryotoxic effects of thalidomide and related compounds were investigated in rabbits of the following strains: Chinchilla, New Zealand White and Black Himalayan. For the most part, Chinchilla rabbits were used and what follows is a description of the general testing procedure using this strain. Adult female Chinchilla rabbits weighing 3 to 4 kg, maintained on pellets of Diet SG-1 (Joseph Rank) and water *ad libitum*, were mated with New Zealand White males (weight 4 to 5 kg). Mating day was considered as day zero and the compound being tested was given daily by means of a stomach tube on days 6 to 15 of pregnancy. In some experiments the compounds were injected intravenously into the ear vein as a solution in 10 ml. of 0.1 M-phosphate buffer, *p*H 7.4. On the 28th day of pregnancy the animals were killed, the uterus was opened, viable foetuses were removed and the presence of any resorption sites was noted. Each foetus was removed, weighed and examined for gross external malformations. In some cases the foetuses were examined by X-ray techniques.

Sedative action. Thalidomide extends the hexobarbitone-induced sleeping time of mice (Somers, 1960) and of rats (Smith, Williams & Williams, 1962). To determine the effect of the various metabolites upon this sleeping time, female rats (160±10 g) were given the metabolites orally or by intraperitoneal injection, 30 min before an intraperitoneal injection of hexobarbitone sodium (70 mg/kg). The sleeping time was estimated from the time of loss of the righting reflex until recovery.

RESULTS

Glutamine synthetase. Using rat brain glutamine synthetase the formation of γ-glutamylhydroxamate was linear over the first 30 min of the reaction, after which the rate of synthesis slowed down. The enzyme was characterized by its inhibition by sodium fluoride and

TABLE 1

EFFECT OF THALIDOMIDE AND ITS HYDROLYSIS PRODUCTS ON RAT BRAIN GLUTAMINE SYNTHETASE

Glutamine synthetase activity is expressed as the amount of γ-glutamylhydroxamate (in μmoles) formed per g of brain powder per hr. The range for the controls was 960–1,250 μmoles/g/hr

Compound tested	Amount of test compound added to incubate (μmoles)	Glutamine synthetase activity (μmoles/g/hr)
Controls	—	1,105
Thalidomide	0·22	1,010
α-(*o*-Carboxybenzamido)glutarimide	10	1,130
2-Phthalimidoglutaramic acid	50	1,060
4-Phthalimidoglutaramic acid	50	1,100
2-Phthalimidoglutaric acid	20	1,000
2-(*o*-Carboxybenzamido)glutaramic acid	50	1,085
4-(*o*-Carboxybenzamido)glutaramic acid	50	875
2-(*o*-Carboxybenzamido)glutaric acid	15	275
Methionine sulphoxide	25	720
Sodium fluoride	0·125	232·5

methionine sulphoxide, two known inhibitors of this enzyme (Elliott, 1951). Of the compounds tested as inhibitors, only 2-(*o*-carboxybenzamido)glutaric acid showed significant activity (Table 1). Thalidomide and the other metabolites investigated were inactive except possibly 4-(*o*-carboxybenzamido)glutaramic acid, which was weakly active. The tricarboxylic acid, 2-(*o*-carboxybenzamido)glutaric acid, was more effective as an inhibitor than methionine sulphoxide, and 50% inhibition occurred at a concentration of 5 μmoles/ml. The relation between concentration of the tricarboxylic acid and extent of inhibition of glutamine synthetase activity is shown in Fig. 1.

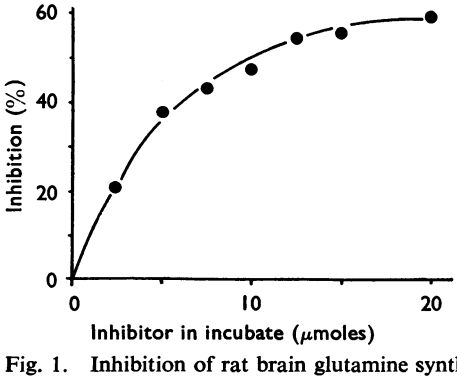

Fig. 1. Inhibition of rat brain glutamine synthetase by 2-(*o*-carboxybenzamido)glutaric acid. Ordinate: inhibition (%); abscissa: μmoles of inhibitor in the incubate.

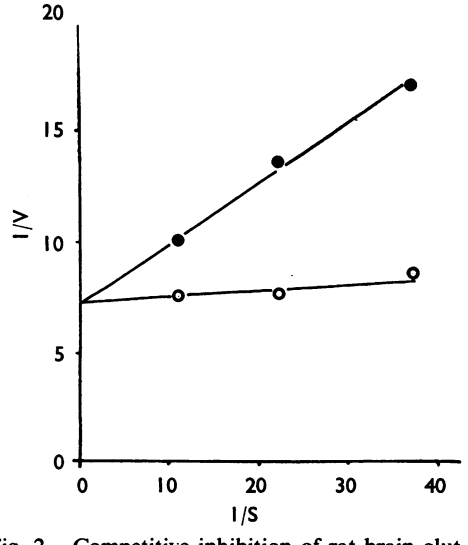

Fig. 2. Competitive inhibition of rat brain glutamate decarboxylase by 2-phthalimidoglutaric acid. Ordinate: 1/V (V = velocity of reaction in μl. of carbon dioxide per hr per flask $\times 10^3$); abscissa: 1/S (S = molar L-glutamate concentration). O——O Control; ●——● 2-phthalimidoglutaric acid.

Glutamate decarboxylase. In the experiments with glutamate decarboxylase of rat brain there was a linear relationship between output of carbon dioxide and time of incubation. In the absence of L-glutamate as substrate there occurred a low rate of release of carbon dioxide (0.59 μmoles/hr/100 mg wet brain) due to endogenous respiration. No increase in carbon dioxide release was evident following the addition of thalidomide or its hydrolysis products, indicating that these substances are not decarboxylated by this enzyme preparation. L-Glutamic acid was decarboxylated at the concentrations used (2.68, 4.46 and 8.92×10^{-2} M), with carbon dioxide release of 2.15, 2.44 and 2.49 μmoles/hr/100 mg wet tissue, respectively. Table 2 shows the effects, expressed in terms of percentage activity of the control, of thalidomide and its metabolites on glutamate decarboxylase activity. The (+)-, (−)- and (±)-forms of thalidomide, 2-phthalimidoglutaramic acid, 4-phthalimidoglutaramic acid, 2- and 4-(*o*-carboxybenzamido)glutaramic acids, DL-glutamine, L-glutamine and phthalic acid did not significantly change the rate of decarboxylation of L-glutamate. However, 2-(*o*-carboxybenzamido)glutaric acid, D-glutamic acid and

TABLE 2

EFFECT OF THALIDOMIDE AND ITS METABOLITES ON RAT BRAIN L-GLUTAMIC DECARBOXYLASE

All compounds were tested at a final concentration of 8×10^{-3} M except (±)-, (−)- and (+)-thalidomide which were used at $0{\cdot}59 \times 10^{-4}$, $0{\cdot}21 \times 10^{-3}$ and $0{\cdot}16 \times 10^{-3}$ M respectively

Compound tested	Activity (% of control) at a substrate concentration of		
	0·0268 M	0·0446 M	0·0892 M
Control	100	100	100
(±)-Thalidomide	93	104	94
(−)-Thalidomide	95	100	110
(+)-Thalidomide	100	94	108
2-Phthalimidoglutaramic acid	95	90	99
4-Phthalimidoglutaramic acid	97	96	110
2-Phthalimidoglutaric acid	48	55	75
2-(*o*-Carboxybenzamido)glutaramic acid	100	95	99
4-(*o*-Carboxybenzamido)glutaramic acid	98	95	98
2-(*o*-Carboxybenzamido)glutaric acid	75	72	86
DL-Glutamine	102	96	104
L-Glutamine	107	101	100
D-Glutamic acid	70	73	109
Phthalic acid	92	—	99

2-phthalimidoglutaric acid inhibited the decarboxylation of L-glutamate. The latter substance was particularly effective, and the nature of its inhibitory effect was further examined. Fig. 2 shows a plot of 1/S (where S is the molar L-glutamate concentration) versus 1/V (where V is the velocity of the reaction expressed as μl. of carbon dioxide per hr per flask $\times 10^3$). It can be seen that the ordinate intercepts given by the slopes of the reactions with and without inclusion of 2-phthalimidoglutaric acid are the same, except that the slope of the reaction has changed, indicating the competitive nature of the inhibition.

Glutamate dehydrogenase. Rat brain glutamate dehydrogenase was characterized by the effects of two known inhibitors, glutaric acid and isophthalic acid. Four of the metabolites of thalidomide interfered with the activity of this enzyme, namely DL-glutamic acid, phthalic acid, 2-(*o*-carboxybenzamido)glutaric acid and 4-(*o*-carboxybenzamido)-glutaramic acid (Table 3). Other metabolites and thalidomide were inactive. Several

TABLE 3

EFFECT OF THALIDOMIDE AND ITS METABOLITES ON RAT BRAIN GLUTAMATE DEHYDROGENASE ACTIVITY

Brain glutamate dehydrogenase activity is expressed in μmoles of $NADH_2$ formed per g of protein per min.
* In 3 ml. of incubate

Compound tested	Amount of test compound* (μmoles)	Brain glutamate dehydrogenase activity (μmoles/g/min)	Activity relative to control (%)
First controls	—	4·55	100
Thalidomide	0·10	4·52	99·5
2-Phthalimidoglutaramic acid	15	4·10	90
4-Phthalimidoglutaramic acid	15	3·65	80
2-Phthalimidoglutaric acid	15	4·45	98
2-(*o*-Carboxybenzamido)glutaramic acid	15	4·20	92
4-(*o*-Carboxybenzamido)glutaramic acid	15	3·40	75
2-(*o*-Carboxybenzamido)glutaric acid	15	3·05	67
Second controls	—	5·45	100
L-Glutamine	60	4·80	88
DL-Glutamic acid	30	2·95	54
Isophthalic acid	15	1·64	30
Phthalic acid	60	3·55	66
Glutaric acid	15	1·38	26

inhibitors of this enzyme have been described by Caughey *et al.* (1957), who partially defined some of the structural requirements necessary in an inhibitor. Thus glutarate, β-ketoglutarate and isophthalic acid are powerful inhibitors, whilst *o*-substituted benzoic acids are inactive or only weakly active as in the case of phthalic acid.

Embryotoxic effect of thalidomide and related compounds in the rabbit. The oral administration of thalidomide (150 mg/kg) to pregnant Chinchilla, New Zealand White and Black Himalayan strains of rabbit from the 6th to the 15th day of pregnancy caused malformation of some of the offspring and an increase in the number of resorption sites (Table 4). The types of malformation seen were similar to those described by Somers (1962) and Felisati (1962) and usually involved the skeleton. The front legs were foreshortened due to a reduction in the long bone formation of the radius and ulna. Both paws often exhibited a characteristic " hook-like position." The hind limbs also manifested malformation involving a shortening and twisting of the tibio-fibula. In one foetus cranioschisis was present. X-ray examination of some of the malformed foetuses showed that in some instances the ossification centres of the carpus and metacarpus were atrophic, and sometimes these centres were missing. Similarly, in deformed hind limbs, the tarsus and metatarsus ossification centres were atrophic or absent.

The oral administration to pregnant rabbits of several of the hydrolysis products of thalidomide failed to produce embryotoxic effects, except that α-aminoglutarimide induced a small increase in the number of resorptions. The intravenous administration of 2-phthalimidoglutaric acid, 2-(*o*-carboxybenzamido)glutaramic acid and 2-(*o*-carboxybenzamido)glutaric acid similarly failed to evoke any apparent foetal damage.

Effect of L- *and* DL-*glutamine on the teratogenic activity of thalidomide.* It is possible that thalidomide produces its embryotoxic effects by blocking the synthesis of glutamine, since the enzyme glutamine synthetase is inhibited by 2-(*o*-carboxybenzamido)glutaric acid, one of the hydrolysis products of the drug. This possibility was investigated by

TABLE 4

SUMMARY OF TERATOGENIC TESTS IN RABBITS

C = Chinchilla; NZ = New Zealand White; BH = Black Himalayan. Days refer to length of pregnancy. I.v.= intravenous

Compound	Treatment	No. of animals	Implantations	Resorptions	Normal foetuses	Malformed foetuses
Controls	—	6 (C)	40	3	37	0
Thalidomide	150 mg/kg orally on days 6–15	4 (C)	29	14	7	8
4-Phthalimidoglutaramic acid	150 mg/kg orally on days 8–16	3 (NZ)	19	3	16	0
2-Phthalimidoglutaramic acid	150 mg/kg orally on days 8–16	2 (NZ)	19	2	17	0
	150 mg/kg orally on days 7–15	3 (C)	29	3	26	0
2-Phthalimidoglutaric acid	3·3 mg/kg i.v. in 0·1 M-phosphate buffer, *p*H 7·4, on days 6–14	2 (NZ)	19	1	18	0
	150 mg/kg orally on days 7–15	3 (C)	29	3	26	0
4-(*o*-Carboxybenzamido)glutaramic acid	100 mg/kg orally on days 8–13	3 (BH)	15	0	15	0
2-(*o*-Carboxybenzamido)glutaramic acid	5 mg/kg i.v. on days 6–16	2 (NZ)	20	3	17	0
2-(*o*-Carboxybenzamido)glutaric acid	15 mg/kg i.v. on days 6–14	1 (NZ)	11	0	11	0
	100 mg/kg orally on days 8–13	1 (BH)	4	0	4	0
α-Aminoglutarimide hydrochloride	150 mg/kg orally on days 6–10 and 75 mg/kg on days 11–15	3 (C)	26	5	21	0

TABLE 5

EFFECT OF L- AND DL-GLUTAMINE ON THE EMBRYOTOXIC ACTIVITY OF (±)-THALIDOMIDE IN RABBITS

The rabbits were Chinchilla strain

Compound	Treatment	No. of animals	Implantations	Resorptions	Normal foetuses	Malformed foetuses
Control	—	3	16	1	15	0
Thalidomide	150 mg/kg orally on days 6–15 of pregnancy	5	39	16	15	8
L-Glutamine	330 mg/kg orally on days 7–15 and 0·1% in drinking water on days 5–17	5	41	2	39	0
DL-Glutamine	As for L-glutamine	5	57	6	51	0
Thalidomide + L-glutamide	L-Glutamine, 330 mg/kg, orally on days 7–15 and 0·1% w/v in drinking water on days 5–17; thalidomide, 150 mg/kg, on days 7–15	3	23	7	10	6

TABLE 6

THE EFFECT OF THALIDOMIDE AND ITS METABOLITES ON THE HEXOBARBITONE-INDUCED SLEEPING TIME IN RATS

All compounds were administered by intraperitoneal injection except for thalidomide which was given orally. Phthalic acid was dissolved in 1 equiv of sodium bicarbonate before its injection. Sleeping times are mean values obtained for groups of eight to ten rats used for each compound

Compound	Dose (mg/kg)	Sleeping time (min)
Controls (first group)	—	23
Thalidomide	200	41
4-Phthalimidoglutaramic acid	300	24
2-Phthalimidoglutaramic acid	300	20
4-(*o*-Carboxybenzamido)glutaramic acid	250	24
2-(*o*-Carboxybenzamido)glutaramic acid	330	21
Phthalic acid	300	20
Controls (second group)	—	24
α-Aminoglutarimide hydrochloride	400	37

attempting to block the embryotoxic effects of thalidomide *in vivo* by previously treating pregnant animals with L-glutamine (Table 5). Neither L- nor DL-glutamine were teratogenic at the dose levels used, although the DL-amino acid appeared to induce a small increase in the incidence of resorptions. In investigating the potential protective action of L-glutamine, the amino acid was fed to pregnant rabbits commencing on the 5th day of pregnancy, 2 days before dosing with thalidomide. Following this, the amino acid was administered both as a single daily oral dose and as a solution in the drinking water. With this regime it was hoped to increase the endogenous stores of L-glutamine, and to ensure an even and continuous intake of the amino acid during the experiment. L-Glutamine, however, did not alter the embryotoxic effects of thalidomide.

Sedative activity. Regarding central nervous depressant activity, the only compound apart from thalidomide to show an extension of hexobarbitone-anaesthesia in rats was α-aminoglutarimide (Table 6). The five other metabolites tested, namely 2- and 4-phthalimidoglutaramic acid, 2- and 4-(*o*-carboxybenzamido)glutaramic acid and phthalic acid, were inactive in this test. The other metabolites, that is α-(*o*-carboxybenzamido)-glutarimide, 2-phthalimidoglutaric acid and 2-(*o*-carboxybenzamido)glutaric acid, were not tested and it seems probable that the amino acids, glutamine, isoglutamine and glutamic acid, are inactive in this respect.

DISCUSSION

Three principal types of biological activity have been attributed to thalidomide, namely embryotoxicity, sedative activity and neurotoxicity; the last is the ability to induce peripheral neuropathies in man following repeated dosage with the drug. The purpose of these investigations was to attempt to find out something about the biochemical mechanisms by which the drug produces its effects. In several respects the biological properties of this drug appear somewhat anomalous. Thus, it is toxic to the embryonic tissues of some species such as the rabbit (Somers, 1962) and the chicken (Kemper, 1962) but not to others such as the cat and hamster (Somers, 1963). Furthermore, thalidomide is distinguished by its lack of toxicity to the adult animal. Most other chemical teratogens, unlike thalidomide, appear to be toxic to both embryo and adult (Murphy, 1960). Different species also vary in their reaction to the central nervous depressant properties of the drug.

The mouse, rat and guinea-pig are sedated by large doses of the drug (Somers, 1960), whereas the rabbit is not. Man, however, appears to be a species uniquely sensitive to the sedative and embryotoxic effects of the drug, since the dose used clinically is relatively low (100 to 200 mg). Our approach to these problems was to try to find out whether the drug itself or a metabolite were responsible for the biological effects, since it is important to determine the active drug species before the question of mechanism can be effectively considered.

Studies on the biological properties of thalidomide, however, are complicated by its inherent instability under physiological conditions, for at *p*H 7.4 and 37° C it breaks down spontaneously to some twelve compounds. Similarly, studies on several of the metabolites themselves are complicated because they are also unstable.

Since thalidomide and most of its metabolites are related to glutamine and glutamic acid, the effects of the drug could be the result of an interference with the metabolism of these amino acids, for the importance of these two compounds in intermediary metabolism is well established (Meister, 1956). Glutamine is found in relatively high concentrations in a variety of tissues and is utilized in numerous reactions. Thus, it is involved in the biosynthesis of glucosamine-6-phosphate (important in connective tissue biosynthesis), of purines (for nucleic acid synthesis), of histidine and of NAD. Both glutamic acid and glutamine are involved in protein synthesis and they are also important in the biosynthesis of new amino acids by transamination. The detoxication of ammonia is effected by its combination with glutamic acid to give glutamine.

Glutamic acid is present in relatively high concentration in brain where it serves as a ready source of energy. Brain tissue also contains relatively large amounts of the enzyme glutamate decarboxylase which converts glutamic acid to γ-aminobutyric acid by decarboxylation. The role of this amino acid in neural metabolism is not yet clear though it has been suggested that it may play the role of a central neurohormone (Baxter & Roberts, 1960).

Some of the metabolites of thalidomide, though not the drug itself, have been found to inhibit moderately *in vitro* the activity of three rat brain enzymes concerned with glutamate metabolism. Thus 2-(*o*-carboxybenzamido)glutaric acid inhibited both glutamine synthetase and glutamate decarboxylase. 2-Phthalimidoglutaric acid inhibited rat brain glutamate decarboxylase *in vitro* in a competitive manner, and the enzyme, glutamate dehydrogenase, was inhibited by four compounds, namely 4-(*o*-carboxybenzamido)-glutaramic acid, 2-(*o*-carboxybenzamido)glutaric acid, DL-glutamic acid and phthalic acid. The question arises as to whether the inhibitory action of these metabolites on glutamate-metabolizing enzymes is significant in relation to the central nervous depression evoked by thalidomide. At present this is difficult to assess, since little is known about the function of these amino acids in the brain, particularly how alterations in their metabolism influence behaviour. None of the metabolites investigated, apart from α-aminoglutarimide, exhibited central nervous depressant activity when given to rats. This compound was active, but its depressant activity was of a weaker order compared to that of thalidomide. However, when considering these results it is important to bear in mind the physicochemical properties of the majority of the metabolites. With the exception of α-aminoglutarimide, the metabolites are all strong acids which will be highly ionized at physiological *p*H values, and

consequently they would penetrate cells only with difficulty. When administered, these compounds may not accumulate at the site of action in sufficient quantity to evoke a biological response. Thalidomide, on the other hand, because of its low polarity and lipid solubility should enter cells more easily, but once inside the cell it will break down spontaneously to its hydrolysis products, some of which may be biochemically active. In this sense one might conceive thalidomide functioning as a latent carrier form, capable of passing into cells and then undergoing spontaneous degradation to biologically active metabolites. These compounds, by influencing the metabolism of brain glutamine and glutamate, may induce behavioural changes. It is also possible that thalidomide (or its metabolites) are centrally active by a mechanism not involving these amino acids.

Disturbances in glutamate metabolism may be an important factor in drug-induced peripheral neuropathies. Thus the persistent inhibition of glutamate-metabolizing enzymes resulting from the chronic clinical use of thalidomide may result in neuropathic reactions. This possibility is at present impossible to assess because our knowledge of the biochemistry of peripheral neuropathies is very meagre.

Whether a relationship exists between the embryotoxic action of thalidomide and inhibition of glutamate-metabolizing enzymes is difficult, as yet, to assess. In rabbits, the embryotoxic effects of the drug are exerted during the 7th to 12th day of pregnancy (Felisati & Nodari, 1963). In this species this corresponds to the period of morphogenesis, shortly after implantation has occurred. It is possible that the embryopathic effect of the drug may arise from a direct action of the drug on the embryo or it may be a secondary effect arising from an interaction of the drug with the mother. This point remains to be settled, but it is provocative that thalidomide and some of its hydrolysis products are known to be present in the rabbit embryo at the critical period, when the drug is administered to the mother (Fabro, Schumacher, Smith & Williams, 1964).

During the period of morphogenesis when the main organ structures are undergoing differentiation there occurs a rapid increase in the synthesis of new nucleic acids, proteins and enzymes required for cellular differentiation and development. Very little is known about the role of glutamine and glutamic acid during embryonic morphogenesis (see Deuchar, 1962). If they are of importance it is possible that an inhibition of the enzymes concerned with their metabolism may be of significance. Thus alteration of the activity of glutamate-metabolizing enzymes may disturb growth and development to the extent of leading to a malformation of the newborn animal. Teratogenic effects induced by glutamine analogues have been described by Murphy (1960). The compounds *O*-diazoacetyl-L-serine (azaserine) and 6-diazo-5-oxo-L-norleucine are both teratogenic in the rat and they antagonize glutamine in some systems.

Attempts to prevent the embryotoxic effects of thalidomide by previous treatment with L-glutamine were unsuccessful. This might suggest that if the teratogenic effects of thalidomide are related to an inhibition of glutamate-metabolizing enzymes then the lesion is not at the point of glutamine biosynthesis, but a block at the utilization of this amino acid cannot be excluded.

SUMMARY

1. Since thalidomide gives rise in the body to twelve hydrolysis products, it is important to evaluate the biological activity of these compounds, for the activity of the parent drug

may be mediated by one or more of these metabolites. For this purpose a number of the hydrolysis products have been evaluated in biological tests for embryotoxic activity in rabbits, central nervous depressant activity in rats and effects on the glutamate decarboxylase, glutamate dehydrogenase and glutamine synthetase of rat brain.

2. For evaluation of embryotoxic activity the compound being tested was administered orally or parenterally to pregnant rabbits, usually on days 7 to 15 of pregnancy. On the 28th day the rabbit was killed and the uterus examined for resorption sites and viable foetuses for malformations.

3. The central nervous depressant activity of the metabolites was evaluated by observation of changes in the overt behaviour of rats after the administration of the compounds and also by changes in hexobarbitone-induced hypnosis.

4. Rat brain was used as a source of the enzymes, glutamate decarboxylase, glutamate dehydrogenase, and glutamine synthetase. Glutamate decarboxylase activity was followed manometrically, whilst glutamate dehydrogenase and glutamine synthetase activities were both measured spectrophotometrically.

5. In contrast to thalidomide, none of the hydrolysis products appeared to be significantly embryotoxic in rabbits.

6. None of the metabolites, apart from α-aminoglutarimide, produced symptoms of overt depression and they had no effect on the hexobarbitone-induced sleeping time in rats. α-Aminoglutarimide was active in these tests and this suggests that the hypnotic activity of thalidomide is related to this particular ring structure.

7. A number of the hydrolysis products were moderately active as inhibitors of some enzymes concerned with glutamate metabolism. Glutamine synthetase was inhibited by 2-(*o*-carboxybenzamido)glutaric acid and to a less extent by 4-(*o*-carboxybenzamido)-glutaramic acid. Glutamate dehydrogenase was weakly inhibited by these two compounds and by phthalic acid, but more effectively by DL-glutamic acid. 2-Phthalimidoglutaric acid was a competitive inhibitor of glutamic decarboxylase.

8. The possible significance of these results in relation to the biological properties of the drug are discussed.

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