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MICROBIOLOGICALLY DETERMINED PANTOTHENIC ACID AND NICOTINIC ACID CONTENT OF CHICK EMBRYOS AFTER TREATMENT WITH THALIDOMIDE AND OF RAT FETUSES, NEWBORNS AND PLACENTAS FROM MOTHERS TREATED WITH THALIDOMIDE

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Abstract—The pantothenic acid and nicotinic acid contents of the liver of chick embryos were determined microbiologically after the eggs had been injected with 2·5 mg or 1·0 mg of thalidomide before hatching. Pantothenic acid values in the thalidomide chicken embryos were about 1/3–1/2 the values in the controls. Quantities of nicotinic acid were 16–17 times greater in the thalidomide groups than in the controls. Both differences are statistically significant. Analyses were also made of the liver of rats treated with thalidomide during pregnancy and of their fetuses, the liver of newborns and placentas. These revealed no differences from the corresponding organs of the controls. An increased incidence of malformations could not be demonstrated in either chickens or rats. In spite of this, however, the results of the vitamin analyses are correlated to the teratogenic action of thalidomide, and their significance in the pathogenesis of the malformations is discussed on the basis of earlier investigations which show the importance of pantothenic acid and nicotinic acid in fetal development.

INTRODUCTION

IN THE literature on thalidomide malformations attention has, from the outset, been given to the possible role of the vitamins of the B-group in the pathogenesis. One of the first to voice the possibility was Kemper,[1–3] and he was quickly followed by others.[4,5] At the same time positive experiences were reported from therapy with the B-vitamins in neuritis[6] and in lesions of mucous membranes[7] after treatment with thalidomide. Experimental investigations have tended to confirm these reports. Thalidomide inhibits the growth of the flagellates *Euglena gracilis*, *Ochromonas malhamensis* and *Ochromonas danica* and the ciliate *Tetrahymena piriformis*, and this inhibition is reversed by nicotinic acid, nicotinamide and nicotinamide adenine nucleotide,[8–10] but no other B-vitamins have any effect. On the other hand, the growth of riboflavin-dependent *Lactobacillus delbrueckii* is accelerated by thalidomide, while on *Lactobacillus plantarum* dependent on niacin and biotin, it has no effect.[11–13]

No analytical data of the effect of thalidomide on the vitamins of the B-group have previously been published. The present paper reports the results of pantothenic acid and nicotinic acid determinations from chick embryos after treatment with thalidomide and of rat fetuses, newborns and placentas from mothers treated with thalidomide.

MATERIALS AND METHODS

Experiments with chick embryos

Fresh white Leghorn eggs* were used, and thalidomide† suspended in 0·1 ml of sterile physiological saline was injected into the yolk sac of fertilized eggs according to the technique of Cravens and Snell.[14] The eggs were divided into three groups of 100, according to the quantity of thalidomide injected: the first group received 2·5 mg, the second 1·0 mg, and the third forming the control group, no thalidomide but 0·1 ml of sterile physiological saline. The thalidomide was sterilized in powder form at 140° for 2 hr. Hatching was begun immediately after the injection, and the eggs were examined by transmitted light at intervals of 3–4 days to detect infertile eggs and dead embryos. On the 15th day of hatching the embryos were removed, cleaned, weighed and studied macroscopically for anomalies. Embryos with malformations and 9–10 others from each group, taken at random, were subjected to vitamin analyses. A piece of the liver was weighed and was homogenized in a motor-driven glass grinder with a Teflon pestle in 5·0 ml of distilled water. Pantothenic acid and nicotinic acid were determined on this suspension after extraction procedures.

Experiments with rats

Adult white laboratory rats, previously unmated, and weighing 180-250 g were used as test animals. The females were mated by placing them in the same cage with males (6–9 females/1–3 males), and the beginning of gravidity was ascertained by observing the vaginal plug.

The animals were fed the normal diet.‡ Thalidomide was given in the drinking water, 10 g/l; the thalidomide was dissolved in dilute NaOH, neutralized with HCl, and tap water was added up to the desired volume. Both food and drinking water were given *ad libitum*. The daily consumption of drinking water was observed and found to be about 7 ml per rat, giving about 70 mg of thalidomide per rat per day.

The animals were killed by decapitation. Organs and fetuses subjected to the vitamin analyses were weighed and ground in 5·0 ml of distilled water. The newborns were also weighed. All fetus and newborns were studied macroscopically for malformations.

The animals were divided into three groups as follows:

Group 1. This consisted of nine female rats mated with untreated male rats and given thalidomide during pregnancy. One of these rats was killed on the 14th day of gravidity and another on the 17th. A piece of the mothers' liver, the placentas and the fetuses as a whole were analysed for the pantothenic acid and nicotinic acid content. Seven of the rats were allowed to deliver. The fetuses of two of these rats were subjected to the vitamin analyses and a piece of the liver was analysed.

Group 2. This consisted of nine female and two male rats. The male rats were given thalidomide in drinking water (10 g/l) for 18 days before they were put with the females. The females were given no thalidomide. All the females were allowed to

* Donated by Messrs. Turun Muna Oy, Turku, Finland.

† Kindly placed at our disposal by Messrs. Oy Star Ab, Tampere, Finland.

‡ Whole wheat flour, whole corn flour, whole soybean flour and skimmed milk powder—24 per cent (w/w) each; 2 per cent feed yeast and 2 per cent minerals (Fosfori Terki. Lääke Oy, Turku, Finland). A porridge was made of these ingredients and water, and 25 g of soya oil was added per kilo of dry substance. We are indebted to Messrs. Lääke Oy for providing us with this diet.

deliver, and the deliveries occurred 23–42 days after they were put with the male rat. No vitamin analyses were made from this group.

Group 3. This group consisted of six female rats to serve as controls. They were treated similarly to those in group 1, but the drinking water was without thalidomide. One of the animals was killed on the 15–16th day of gravidity and another on the 17th. Vitamin analyses were made from the corresponding subjects to those in group 1.

Analysis for pantothenic acid and nicotinic acid

The samples were microbiologically analysed for pantothenic acid[15] and nicotinic acid[16] using *Lactobacillus plantarum* as the test organism. The methods are adapted to suit our laboratory conditions.[17] The samples for the pantothenic acid determinations were treated with commercial intestinal phosphatase (Armour Pharmaceutical Company, Ltd, Eastbourne, England) and liver amidase (L. Light and Company, Colnbrook, England) at pH 8·2. After extraction of the fatty acids at pH 4·5 and separation of the proteins, clear liquid was obtained for pantothenic acid determination. The samples for the nicotinic acid determinations were extracted by boiling for 15 min in N/10 HCl under 15 lb pressure in the autoclave.

Statistical treatment

Students' *t*-test was employed in the statistical treatment of the results.

RESULTS

Experiments with chick embryos

Table 1 gives the number of embryos which died during hatching and of those

TABLE 1. NUMBER OF DEAD AND LIVING EMBRYOS IN EGGS TREATED WITH THALIDOMIDE BEFORE HATCHING, AND IN THE CONTROLS. DAYS READ FROM THE BEGINNING OF HATCHING

Group	Total	Dead embryos				Alive
		4th day*	7th day	11th day	15th day	15th day
2·5 mg thalidomide	100	40	22	1	3	34
1·0 mg thalidomide	100	33	12	11	2	42
Controls	100	28	17	5	2	48

* Includes eggs which failed to develop

which were alive on the 15th day of hatching. The number of living embryos is remarkably small in the control group, but in both groups treated with thalidomide it is even smaller. The difference between the group treated with 2·5 mg thalidomide and the control group is statistically almost significant ($t=2{\cdot}02$, $0{\cdot}05 > P > 0{\cdot}02$). In weight of embryos there is no statistical difference between the various groups (Table 2).

Macroscopical examination of the embryos revealed the following malformations:

Group of 2·5 mg thalidomide: One embryo with omphalocele and one other with a slight reduction in long-bone formation of the feet. Measured from heel to toes the length of both feet of this embryo was 17 mm, while the others in the same group had a corresponding length of $21{\cdot}0 \pm 1{\cdot}3$* mm.

* Standard deviation.

Group of 1·0 mg thalidomide: One embryo with microphthalmus on the left side.

Control group: One partially dicephalic embryo, which had two beaks, three eyes and cerebral hernia.

The results of the pantothenic acid and nicotinic acid analyses are presented in Table 2. The content of pantothenic acid in the liver of embryos in the control group

TABLE 2. WEIGHT OF CHICKEN EMBRYOS ON THE 15TH DAY OF HATCHING, AND PANTOTHENIC ACID AND NICOTINIC ACID CONTENT OF THE LIVER OF THESE EMBRYOS

Group	Number	Weight of embryos* (g)	Pantothenic acid in liver (μg/g)	Nicotinic acid in liver (μg/g)
2·5 mg thalidomide	12	7·20 ± 0·74†	22·16 ± 11·08†	167·12 ± 23·76†
1 mg thalidomide	11	6·85 ± 0·88	14·63 ± 5·42	177·99 ± 50·15
Controls	10	7·83 ± 1·13	40·38 ± 16·53	10·58 ± 2·73

* Includes all embryos alive on the 15th day of hatching
† Standard deviation

is considerably greater than in the embryos treated with thalidomide. The differences are statistically significant: in a comparison between the controls and the group of 2·5 mg of thalidomide, $t = 3{\cdot}08$ and $0{\cdot}01 > P > 0{\cdot}001$; between the controls and the group of 1·0 mg of thalidomide, $t = 5{\cdot}35$ and $P < 0{\cdot}001$

The results of the nicotinic acid analyses are directly contrary to the pantothenic acid values. In the thalidomide groups the nicotinic acid concentration in the liver of embryos is significantly much greater than in the controls. Statistical treatment reveals values $t = 20{\cdot}63$ and $P \ll 0{\cdot}001$ for the group of 2·5 mg of thalidomide, and $t = 10{\cdot}53$ and $P \ll 0{\cdot}001$ for the group of 1·0 mg.

In vitamin analyses embryos with malformations gave in every group similar results to those of the other subjects in the same group. Malformed embryos are included in the tables.

TABLE 3. NUMBER AND WEIGHT OF OFFSPRING IN THREE GROUPS OF RATS WITH FULL-TERM GRAVIDITY

(Rats subjected to vitamin analyses are also included in this table)

Group	Number	Number of offspring total	Number of offspring per mother	Weight of offspring (g)
1. Females treated with thalidomide during pregnancy	6	64	10·7 ± 3·1*	5·152 ± 0·493*
2. Males treated with thalidomide before conception; no thalidomide given to females	8	57	7·1 ± 2·3	5·347 ± 0·857
3. Controls	4	39	9·8 ± 4·6	4·954 ± 0·565

* Standard deviation

Experiments with rats

There are no statistically significant differences in number and weight of fetuses, newborns and placentas between the three groups of rats (Tables 3 and 4). The weight of the fetuses and placentas of the rat treated with thalidomide during pregnancy and

TABLE 4. WEIGHT OF RAT FETUSES AND PLACENTAS, AND PANTOTHENIC ACID AND NICOTINIC ACID CONTENT OF THE LIVER OF PREGNANT RATS (TWO TREATED WITH THALIDOMIDE AND TWO CONTROL RATS), IN THE WHOLE RAT FETUS AND PLACENTAS AT VARIOUS STAGES OF PREGNANCY

	Thalidomide group				Control group			
	Number	Weight (mg)	Pantothenic acid (μg/g)	Nicotinic acid (μg/g)	Number	Weight (mg)	Pantothenic acid (μg/g)	Nicotinic acid (μg/g)
	Duration of pregnancy		14 days				15-16 days	
Liver of mother	1	—	60·85	131·80	1	—	48·86	35·02
Whole fetus	8	314·1±27·1*	4·01±1·18*	20·86±2·55*	9	492·8±43·4*	9·01±7·12*	25·32±13·34*
Placentas	5	199·6±12·7	11·92±2·98	27·21±2·61	5	285·4±17·8	20·56±4·51	26·59±2·70
	Duration of pregnancy		17 days				17 days	
Liver of mother	1	—	40·62	85·17	1	—	57·77	86·30
Whole fetus	9	801·0±46·5	28·35±10·75	18·16±2·42	13	754·6±69·3	21·15±11·46	17·41±0·92
Placentas	9	329·3±47·6	17·58±5·96	27·81±1·90	10	334·7±62·5	17·74±4·98	24·58±1·57

* Standard deviation

TABLE 5. PANTOTHENIC ACID AND NICOTINIC ACID CONTENT IN THE LIVER OF TWO THALIDOMIDE RATS WITH FULLTERM GRAVIDITY AND IN THE LIVER OF TWO CONTROL RATS, AND IN THE LIVER OF THEIR OFFSPRING

	Thalidomide group			Control group		
	Number	Pantothenic acid (μg/g)	Nicotinic acid (μg/g)	Number	Pantothenic acid (μg/g)	Nicotinic acid (μg/g)
Liver of mother	1	50·46	—	1	43·55	—
Liver of newborn	8	44·27±33·80*	92·35±11·38*	8	34·69±29·19*	163·69±122·35*
Liver of mother	1	49·64	198·45	1	29·88	174·59
Liver of newborn	9	45·51±9·86	78·06±6·05	8	36·26±20·80	92·22±15·04

* Standard deviation

killed on the 14th day of pregnancy are somewhat lower than these of the corresponding control rat, but this difference is correlated to the differences in duration of the pregnancy.

The above-mentioned rat, treated with thalidomide and killed on the 14th day of pregnancy, also had three dead, partially resorbed fetuses, which are not included in the tables. These were the only dead fetuses seen in our rat series. Macroscopical investigation revealed no malformations either in the group treated with thalidomide during pregnancy or in the controls. One of the female rats whose partners had been given thalidomide before conception had one offspring with meningocele. Other offspring from this mother were not available for investigation because of cannibalism. This litter has been excluded from the tables.

Analyses for pantothenic acid and nicotinic acid (Tables 4 and 5) do not reveal any differences between the group treated with thalidomide during pregnancy and the controls. In rats killed on the 14th day of pregnancy (thalidomide-treated) and on the 15-16th day (control), the quantities of analysed vitamins are not comparable because of the difference in the duration of pregnancy.

DISCUSSION

In the present series of chick embryos, the teratogenic effect of thalidomide was not evident. The few anomalies found closely resemble those described by others in chick embryos,[3, 18–21] but the present control group also included one malformated embryo. Our findings coincide with those of Goerttler[22] and Verret and McLaughlin.[23] The latter emphasize the importance of the substance in which the thalidomide is suspended or dissolved. Kemper[3] has mentioned the differences in stock and in the care of the parent animals as a possible explanation of variable malformation rate.

It is generally accepted that thalidomide induces fetal resorptions in the rat but no malformations.[24–32] The present observations agree with this. In the group in which the males were treated with thalidomide before conception, one fetus with meningocele was observed. No great significance can, however, be attached to this, because of the smallness of the size of our material.

Results of the vitamin analyses show significantly lower concentrations of pantothenic acid in the liver of thalidomide-treated chick embryos, while nicotinic acid concentrations are 16–17 times greater than in the controls. These are interesting findings, but on the basis of the present study it is impossible to say what correlation exists between these two facts. They may be parallel findings, or one may be a primary phenomenon and the other a consequence. Does thalidomide inhibit the uptake of pantothenic acid in the chick embryo, or does it accelerate the breakdown and utilization of pantothenic acid? Does thalidomide block the utilization of nicotinic acid, causing its accumulation in the liver? (It must also be remembered that chick embryo is able to synthetisize nicotinic acid.[33]) The answers to questions such as these should explain the significance of the present findings. It was also possible that the microorganism used in our determinations of pantothenic acid and nicotinic acid could be affected by thalidomide or its metabolites. With several experiments using thalidomide in varying concentrations in suspension and in solution, we have ascertained that the growth of the test strain of *Lactobacillus plantarum* used in the present study is not

affected by thalidomide in any way, at least not under the conditions prevailing in the determinations.

In the same way we have studied the effect of thalidomide on other micro-organisms, including several strains of *Corynebacterium*, *Bacillus subtilis*, *Proteus*, *Klebsiella*, *Escherichia*, *Shigella Salmonella*, *Pseudomonas*, *Staphylococcus*, *Streptococcus*, *Diplococcus pneumoniae* and *Candida*, and on HeLa cells. Thalidomide was found to be inert in these experiments.

Similar findings concerning *Lactobacillus plantarum*[10–13] and other bacteria[10,34] and leucocyte cultures[35] have also been reported by others. These observations do not, however, exclude the possibility that the test strain is affected by the metabolites of thalidomide, which are largely glutamic acid derivatives[36–39]. This is unlikely, if not impossible, since the results of pantothenic acid and nicotinic acid determinations in chick embryos are contradictory, and in the determinations of both vitamins the same micro-organism was used.

Vitamin analyses of specimens from rats do not reveal any effect of thalidomide. This can be attributed either to the fact that thalidomide produces no malformations in the rat or, possibly, to the instability of thalidomide in solutions.[39]

Whatever the explanation of thalidomide interaction in the metabolism of pantothenic acid and nicotinic acid it may be assumed that the present findings are related to the malformations caused by thalidomide. Both these vitamins are known to be important factors in fetal development. This has been demonstrated in several experimental investigations. When pregnant rats are fed on a diet deficient in pantothenic acid a high incidence of malformed fetuses is observed.[40–49] The malformations include cerebral, visceral, dermal and skeletal anomalies. Deficiency caused by specific pantothenic acid antagonists such as ω-methyl pantothenic acid[47,49] and pantoyltaurine[50] has similar effects. Beak and skeletal anomalies in chicks have been produced by injecting the eggs with 6-aminonicotinamide, a nicotinic acid antagonist.[51–53] Corresponding malformations and visceral anomalies have also been produced by this drug in rats[52,54,55] and mice.[56,57] The toxicity of 6-aminonicotinamide could be reversed by the simultaneous injection of nicotinamide.[52,57] Findings that are extremely interesting in the light of our present results have been reported by Lefebvres-Boisselot[43] and Hansborough.[58] The former, in experiments with rats, has observed that the teratogenic effects of the pantothenic acid deficient diet was intensified by the addition of nicotinic acid. Hansborough has produced malformations in chick embryos with an increased amount of nicotinic acid in the egg.

Despite the fact that the teratogenic effect of thalidomide could not be demonstrated in the present series, the analytical data concerning pantothenic acid and nicotinic acid reveal an interesting aspect of the metabolic effects of this drug. If one assumes that the primary effect of thalidomide is pantothenic acid deficiency, this alone might explain the anomalies. In the rat, the presence of 20–25 μg of pantothenic acid per day during pregnancy causes a high incidence of malformed fetuses, while a daily dose of 50 μg is sufficient for undisturbed development.[44,46] If it is accepted that the high concentration of nicotinic acid in the liver of chicks is induced by metabolic block, producing peripheral deficiency, another saitsfactory explanation of the malformations is achieved. The increased quantities of nicotinic acid might also be considered an explanation of teratogenesis. Which is the correct assumption is something that

cannot at present be said, and what other metabolic effects thalidomide has is not yet known.

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