In vitro studies with thiamine tetrahydrofurfuryl disulfide (TTFD) revealed that treatment of the fertilized sea urchin ova produced arrest of cellular division at the metaphase stage (1). It was postulated that this was probably due to interference with interconversion of thiol disulfide responsible for the mitotic mechanism (1, 2). The depressant effect of TTFD on cellular proliferation has also been demonstrated in primary monolayer culture of chick heart cells, hamster and monkey kidney cells (3) and amnion (4) and kidney cells of the human embryo (5).

It has also been shown that in experimental animals (rats and rabbits) there is a more marked accumulation of TTFD in circulating erythrocytes and tissue cells than thiamine (6) and that, in pregnant mice, TTFD·HCl crosses the placental barrier into the foetus (7).

Despite the possible implications of these findings to embryonic development, daily oral administration of TTFD·HCl at 30 and 300 mg/kg (equivalent to 5 to 10 and 50 to 100 times the maximum human therapeutic dosage) to pregnant CF-1 mice and SD rats during the critical stages of organ differentiation failed to produce any significant developmental abnormality (8).

The present report deals with subsequent teratological studies of TTFD·HCl, performed in the rabbit and in the cynomolgus monkey.

MATERIALS AND METHODS

1. Animals

Albino rabbits derived from a closed colony, Tokyo Farm, Takeda Chemical Industries, were maintained in an air-conditioned environment with the temperature of 23±1°C and humidity of 55±5%. They were allowed free access to drinking water and fed a mixture of commercial diets (50% Oriental RC-5 and 50% Funabashi diet). When mated, males and females were, respectively, 12 and 10 months of age and sexually mature.

In another series of experiments a smaller albino rabbit (Nibs), obtained from the Experimental Animals Laboratories of Nippon Institute for Biological Science, was employed. When mated, the males were 10 months of age and the females 7.

The cynomolgus monkeys were imported from Cambodia and the Philippines via the

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Orient Trade Co. After an initial period of quarantine, during which the animals were subjected to veterinary examination for dysentery, tuberculosis and other ailments, the animals were acclimatized to the laboratory conditions for at least 2 months. The monkeys were maintained in individual metal cages and fed a daily ration consisting of 100 g solid diet (Hikari Farm, Takeda Chemical Industries), 130 g sweet potato and half of one apple. 250 ml drinking water was supplied daily. Additionally a 50 mg of Vitamin C supplement was added to the drinking water twice a week.

2. **Mating**

For the rabbits the female was taken to the male and copulation was observed. Following successful coitus, which was considered to indicate day 0 of gestation, the females were transferred to individual cages.

For the monkeys, following the descriptions of Ihara et al. (9) only females showing a regular menstrual cycle of 26 to 33 days were selected. Considering the commencement of the menstrual cycle as day 1 the females were mated on days 11, 12, 13 and 14 of the cycle and day 13 was considered to represent day 1 of gestation. On days 18 and 19 of gestation, pregnancy was verified by induction of ovulation in rats by treatment with monkey urine (10). Subsequently the progress of pregnancy was checked at regular intervals by rectal palpation.

3. **Drug administration**

For administration of the drug, TTFD-HCl was dissolved in distilled water immediately prior to dosing, and administered by means of a stomach tube. Dosages employed, period of treatment and number of animals were as follows:

| Animal      | Daily dosage (mg/kg) | Duration of treatment (days of gestation) | Number of females |
|-------------|----------------------|------------------------------------------|-------------------|
| Albino rabbit | 0                    | 16                                        | 16                |
|             | 300                  | 8 to 16                                   | 10                |
| Nibs rabbit | 0                    | 15                                        | 10                |
|             | 30                   | 8 to 16                                   | 9                 |
|             | 300                  | 8 to 16                                   | 10                |
|             | 500                  | 8 to 16                                   | 5                 |
| Cynomolgus monkey | 30                   | 20 to 29, 21 to 30, 23 to 32              | 7                 |
|             | 300                  | 22 to 31, 23 to 32                        | 2                 |
|             | 500                  | 21 to 30, 22 to 27                        | 3                 |

4. **Teratological parameters**

Albino and Nibs rabbits were sacrificed on day 28 of pregnancy by air embolism or ether anaesthesia. Immediately afterwards the uterus was removed by hysterectomy and incised to determine the number of implantations, living and dead young and resorption sites. Viable young were weighed, examined externally and internally for abnormalities, and sexed. Sample foetuses were cleared and stained with alizarin red S following the method of Dawson,
and skeletons examined for abnormalities and variations.

Pregnant monkeys were delivered by Caesarean Section at about day 100 of gestation. About one hour prior to surgery each animal was injected intra-muscularly with 1 ml Ermetrine1 (Takeda). Anaesthesia was then induced by the intra-muscular injection of 0.8 ml/kg of Ketalar1s' 50 (Sankyo) and a mid-line abdominal incision made for exteriorization of the uterus. An incision was made at the placental site, the amount of amnionic fluid measured and the foetus and placenta manually removed. The placenta was weighed immediately, and then fixed in 10° formalin. The foetus was weighed and examined macroscopically for external and visceral abnormalities. Viscera were then weighed and fixed in Bouin’s fluid for subsequent microscopic examination and the remainder of the foetus was fixed in 95° ethanol for subsequent clearing and staining of the skeleton with alizarin red S to detect skeletal abnormalities and variations.

RESULTS

1. Albino rabbits

During the dosing period the mean weight gain (330 g) of pregnant animals receiving 300 mg/kg, was lower than that (440 g) of controls. Total resorption of all embryos occurred in one of the 10 test animals, and although this tended to increase foetal loss (mortality) for the group, the difference from controls was not statistically significant, nor was litter size affected. Mean foetal weight was not affected by treatment. No external malformations were observed among 61 test young; cyclopia and brachyury (short tail) were observed in 2 of the 133 control young (Table 1).

Skeletal examination revealed abnormalities in 4 of the 125 control foetuses examined, these abnormalities being cyclopia and short tail seen externally, and costal fusion (2 pups) and short pubis (3 pups). Among 61 test foetuses only one showed malformation, this being a defect of the zygomatic arch. In respect of more common skeletal variations there was no significant difference between the incidences of test and control groups (Table 2).

2. Nibs rabbits

At 300 and 500 mg/kg evacuation of soft faeces and reduced food intake were observed from the 6th day of treatment. Two of 5 animals receiving 500 mg/kg, died on the 7th day of treatment, autopsy revealing rupture of the stomach wall along the great curvature, serosanguineous pleural exudate, and increased fragility of the liver. At termination autopsy of the 3 surviving animals revealed thinning of the stomach wall along the great curvature, thickening of the pyloric wall and increased fragility of the liver.

Treatment at 300 and 500 mg/kg was associated with weight loss during the dosing period, followed by partial recovery during the subsequent observation period (Table 1). At 30 mg/kg there was no marked effect on parent animals.

Three of 10 animals treated at 300 mg/kg and 2 of the surviving 3 animals treated at 500 mg/kg showed total resorption of all embryos. Due to the influence of these total resorptions, foetal mortality rates of 43.5% and 72.9% were recorded respectively at 300 and 500 mg/kg; the difference from the control value (10.3%) was statistically significant (P<
| Experimental groups | Albino rabbits | | | Nibs rabbits | | | |
|---------------------|---------------|---|---|----------------|---|---|---|
|                     | Control | TTED·HCl (mg/kg/day) | Control | TTED·HCl (mg/kg/day) | | | |
|---------------------|---------|---------------------|---------|---------------------|---|---|---|
| No. of pregnant rabbits | 16 | 10 | 10 | 9 | 3.17 | 3.13 |
| (pregnant day)       |       | (1)*  |   |       | ±0.114 | ±0.127 | ±0.273 |
| Day 1 (pregnant day) | 3.55 | ±0.064 | 2.79 | ±0.114 | ±0.127 | ±0.113 | ±0.273 |
| Day 8               |         | ±0.084 | 2.96 | ±0.134 | ±0.127 | ±0.095 | ±0.285 |
| Body weight (g ± S.E.) | 3.68 | ±0.165 | 3.07 | ±0.134 | ±0.080 | ±0.133 | ±0.292 |
| Day 17              |         | ±0.134 | 3.46 | ±0.133 | ±0.133 | ±0.133 | ±0.292 |
| Day 28              | 3.99   | ±0.088 | 3.09 | ±0.126 | ±0.080 | ±0.147 | ±0.295 |
|                     | ±0.108 | ±0.126 | ±0.295 | | | | |
| Total No. of implants (Mean number per litter) | 159 (9.9) | 82 (9.0) | 87 (8.7) | 75 (8.3) | 92 (9.2) | 24 (8.0) |
| Implantation sites   | 0 | 0 | 0 | 0 | 31 | 13 |
| Placental remnants   | 16 (16.4) | 11 (25.6) | 3 (10.3) | 4 (9.3) | 7 (43.5)** | 3 (79.2)* |
| Macerated foetuses   | 10 | 10 | 6 | 3 | 2 | 3 |
| No. of lives examined | 133 | 61 | 78 | 68 | 52 | 5 |
| Fecundities          | No. of lives with gross malformations (%) | 2(1.5) | 0 | 2(2.9) | 2(3.8) |
| Cyclopia             | 1(0.7) | 0 | 0 | 0 | 0 | 0 |
| Open eyelid          | 0 | 0 | 0 | 2(2.9) | 0 | 0 |
| Cataract             | 0 | 0 | 0 | 0 | 2(3.8) | 0 |
| Short tail           | 1(0.7) | 0 | 0 | 0 | 0 | 0 |
| Mean body weight (g ± S.E.) | 35.2 ±0.99 | 34.2 ±2.00 | 33.6 ±1.89 | 32.0 ±0.93 | 30.6 ±2.00 | 34.9 |
| Sex ratio (♀/♂ × 100) | 118 | 110 | 472 | 94 | 86 | 66 |

* No. of maternal deaths.
** Significantly different from the control at P<0.01.
Table 2. Type and number of skeletal abnormalities and variations in foetuses of Albino and Nibs rabbits treated orally with TTFD-HCl.

| Experimental groups | Albino rabbits | Nibs rabbits |
|---------------------|----------------|--------------|
|                     | TTFD-HCl Control | TTFD-HCl 300 mg/kg/day | TTFD-HCl Control | TTFD-HCl (mg/kg/day) |
| No. of foetuses examined | 125            | 61            | 68            | 30             | 50 | 5 |
| No. of viable foetuses with skeletal abnormalities (%) | 4(3.2)          | 1(1.6)        | 0             | 0              | 0  |
| No. of viable foetuses with skeletal variations (%) | 50(40.0)        | 20(32.8)      | 7(9.0)        | 13(19.1)       | 27(54.0)* | 0 |
| Abnormalities (%)  | Abnormal zygomatic arch  | 0             | 1(1.6)        | 0             | 0  |
|                    | Fused ribs        | 1(0.8)        | 0             | 0             | 0  |
|                    | Short pubis       | 3(2.4)        | 0             | 0             | 0  |
| Variations (%)     | Cervical ribs     | 1(0.8)        | 0             | 0             | 2(4.0) |
|                    | Asymmetry of sternebrae | 1(0.8)       | 0             | 0             | 0  |
|                    | Lumbar ribs       | 50(40.0)      | 20(32.8)      | 7(9.0)        | 11(16.2) | 26(52.0)* | 0 |
| Numerical variation of lumbar vertebrae | 0              | 0             | 6(7.7)        | 3(4.4)        | 1(2.0) |

* Significantly different from the control at P<0.01.

At 30 mg/kg there were no total resorptions and foetal mortality was comparable to that of control. Mean pup weight of foetuses in all test groups was unaffected.

Abnormalities observed included 2 foetuses with open eye lids at 30 mg/kg, and 2 foetuses with lenticular opacity at 300 mg/kg. Respective malformation rates at 0, 30, 300 and 500 mg/kg were 0 out of 78, 2 out of 68, 2 out of 52 and 0 out of 5 (Table 1).

Skeletal examination revealed no further major malformations in either test or control foetuses, but there was a significant increase in the incidence of pups with extra lumbar ribs (Table 2).

3. Cynomolgus monkeys

In test monkeys treated with 300 and 500 mg/kg, signs of reaction included the evacuation of soft foeces, diarrhoea, and reduced food consumption. During the early period of administration emesis was noted in one out of 3 monkeys treated at 500 mg/kg (Table 3).

Abortion occurred in 2 out of 7 control animals at 47 and 55 days of gestation. The remaining 5 animals carried young foetuses, normal in external appearance and bodyweight at sacrifice. Alizarin staining revealed no abnormalities other than the presence of a cervical rib in 2 young, one of which also had a short 12th rib.

At 30 mg/kg abortion, accompanied by obvious uterine bleeding, occurred in one of the 7 treated monkeys at day 33 of gestation. This animal had experienced abortion once in her previous reproductive process. One monkey showed placental remnant on the day of Caesarean Section and another one animal bore a macerated foetus, normal in external appearance. The remaining 4 monkeys contained viable female young with normal external
### TABLE 3. Maternal findings in pregnant cynomolgus

| Experimental groups | Control Untreated |
|---------------------|-------------------|
| Animal number       | T 45 T-20 T-51 T-9 T-24 T-13 T-8 |
| Treatment (pregnant day) | 3.30 3.45 3.20 3.09 3.03 3.50 2.90 |
| Day 1 (pregnant day) | 3.50 3.50 3.14 2.95 3.03 3.45 2.90 |
| Body weight         | Day 21 3.49 3.50 3.26 3.80 2.90 3.40 2.90 |
| Day 100             | 4.02 3.90 4.40 4.10 4.46 3.10 |
| Feces               | Normal Normal Normal Normal Normal Normal Normal |
| Food intake         | Good Good Good Good Good Poor Poor |
| Vaginal bleeding    | P : 4 P : 1 P : 2 P : 3 P : 1 |
| Previous reproductive history in the laboratory | (C : 1) (C : 1) (C : 2) (C : 1) None None |
|                     | (A : 3) (A : 1) (A : 1) |

P : Pregnancy  C : Caesarean  B : Birth  A : Abortion

### TABLE 4. Foetal findings in pregnant cynomolgus

| Experimental groups | Control Untreated |
|---------------------|-------------------|
| Animal number       | T-45 T-20 T-51 T-9 T-24 T-13 T-8 |
| Live or death       | Live Live Live Live Live Live |
| Sex                 | ♂ ♂ ♂ ♂ ♂ ♂ |
| Body weight (g)     | 135 126 128 139 129 120 |
| Body length (mm)    | 125 121 129 132 131 134 |
| Tail length (mm)    | 122 132 130 141 112 |
| Arm length (mm)     | 85 90 94 93 91 |
| Leg length (mm)     | 89 92 95 103 93 |
| Placental weight (g) | 69 62 57 43 47 |
| Gross observation   | Normal Normal Normal Normal Normal Normal |
| Brain               | 18.1 15.9 14.8 18.2 13.0 |
| Heart               | 0.75 0.78 0.68 1.01 0.70 |
| Lung                | 2.76 2.12 2.41 3.71 2.30 |
| Liver               | 4.35 4.32 3.90 4.74 3.80 |
| Kidney Right        | 0.43 0.29 0.35 0.41 0.32 |
| Kidney Left         | 0.42 0.30 0.36 0.43 0.30 |
| Adrenal gland Right | 0.039 0.020 0.014 0.031 0.020 |
| Adrenal gland Left  | 0.039 0.023 0.010 0.026 0.015 |
| Spleen              | 0.33 0.25 0.26 0.39 0.19 |
| Thymus              | 0.34 0.26 0.30 0.33 0.20 |
| Skeletal observation| Normal Normal Normal CR SR CR |

CR : Cervical rib  SR : Shortening of the 12th rib
monkeys treated orally with TTFD•HCl.

| Treatment (mg/kg/day) | 30 | 300 | 500 |
|----------------------|----|-----|-----|
|                      | T-67 | T-71 | T-47 | T-12 | T-53 | T-48 | T-64 | T-54 | T-26 | T-50 | T-62 | T-3 |
| 21-30                | 21-32 | 21-30 | 20-29 | 21-30 | 21-30 | 23-32 | 22-31 | 23-32 | 21-30 | 22-27 | 21-30 |
| 2.80                 | 3.72 | 3.10 | 4.20 | 2.90 | 4.00 | 3.40 | 2.70 | 3.48 | 3.70 | 3.70 | 3.70 |
| 3.00                 | 3.70 | 3.15 | 4.24 | 3.00 | 4.05 | 3.54 | 2.88 | 3.57 | 3.81 | 3.75 | 3.75 |
| 2.94                 | 3.50 | 3.15 | 4.18 | 3.05 | 4.00 | 3.38 | 2.70 | 3.67 | —   | —   | 3.70 |
| 3.10                 | 2.85 | 5.49 | 3.14 | 4.24 | 4.10 | 3.50 | 3.87 | —   | —   | 4.30 |

| Normal | Normal | Loose | Normal | Normal | Diarrhea | Loose | Diarrhea | Loose | Diarrhea | Loose |
|--------|--------|-------|--------|--------|----------|-------|----------|-------|----------|-------|
| Poor   | Good   | Good  | Good   | Good   | Good     | Poor  | Poor     | Poor  | Poor     | Vomiting Poor |
| P: 2   | P: 4   | P: 1  | P: 1   | P: 2   | P: 1     | P: 2  | P: 1     | P: 1  | P: 2     |

None  None  | (C: 1)  | (C: 1)  | (C: 1)  | (C: 1)  | (C: 1)  | (C: 1)  | (C: 1)  | (C: 1)  | (C: 2) |
A: 1  B: 1  | A: 2    | A: 1    | A: 1    | A: 1    | A: 1    | A: 1    | A: 1    | A: 1    | A: 1    |

monkeys treated orally with TTFD•HCl.

| Treatment (mg/kg/day) | 30 | 300 | 500 |
|----------------------|----|-----|-----|
|                      | T-67 | T-71 | T-47 | T-12 | T-53 | T-48 | T-64 | T-54 | T-26 | T-50 | T-62 | T-3 |
| Dead                |    |     |     |     |     |     |     |     |     |     |     |     |
| Live               | 134 | 98  | 123 | 129 | 65  | 130 | 135 | 126 | 134 | 134 | 135 | 126 |
| Life (33rd day of pregnancy) | (33rd day of pregnancy) | Normal | Normal | Normal | Normal | Normal | Normal | Normal | Normal | Normal | Normal |
| 19.5                | 13.1 | 17.4 | 15.9 | 8.1  | 17.1 | 19.5 | 0.88 |
| 2.71                | 1.42 | 2.76 | 2.94 | 1.24 | 2.43 | 2.35 |
| 3.70                | 3.20 | 4.21 | 4.66 | 2.38 | 4.52 | 4.15 |
| 0.33                | 0.40 | 0.47 | 0.50 | 0.22 | 0.45 | 0.48 |
| 0.32                | 0.39 | 0.46 | 0.49 | 0.22 | 0.43 | 0.40 |
| 0.049               | 0.040 | 0.042 | 0.036 | 0.024 | 0.028 | 0.026 |
| 0.057               | 0.044 | 0.040 | 0.040 | 0.022 | 0.033 | 0.026 |
| 0.21                | 0.34 | 0.33 | 0.55 | 0.20 | 0.60 | 0.25 |
| 0.20                | 0.11 | 0.25 | 0.31 | 0.08 | 0.27 | 0.40 |
| Normal              | CR  | Normal | Normal | CR  | Normal | CR  | CR  |
appearance and bodyweight; alizarin staining revealed no malformations other than the presence of a cervical rib in one of the young.

At 300 mg/kg both monkeys bore viable male young with normal external appearance. The weight of one of these was low and in the second foetus macroscopic and microscopic examination revealed enlarged congested spleen. Alizalin staining revealed no skeletal abnormalities other than the presence of a cervical rib in both.

At 500 mg/kg abortion, accompanied by obvious uterine bleeding, occurred in 2 of the 3 treated monkeys at days 28 and 29 of gestation. The remaining animal bore a female foetus normal in external appearance and bodyweight; alizarin staining revealed the presence of a cervical rib (Table 4).

**DISCUSSION**

Since the demonstration of teratogenicity of thalidomide in the rabbit (11), this species was frequently available for the examination of drug safety on the developing foetuses. Occurrence of spontaneous malformations are also known in the rabbit. The availability of the monkey for the teratogenicity of chemical substance derived from the following reasons (12): 1) Teratogenic agents in man are also teratogenic in monkey, 2) Agents suspected to be embryotoxic in man are also embryotoxic in monkey, though the parallelism is less close than in the case of teratogenicity, 3) Similarities in the anatomical and temporal aspects of embryogenesis between man and monkey, 4) Similarities in structure and function of the placenta between both species, and 5) Similarity of reproductive physiology between human females and female monkeys.

Results of the presently reported studies, with 2 strains of rabbit and with cynomolgus monkeys, indicate that the administration of TTFD•HCl during the critical stage of organ differentiation causes no significant increase in the incidence of foetal malformations, even at dosages exerting adverse maternal reaction (e.g. diarrhoea, retarded weight gain or loss, and anorexia).

Other embryopathic effects, such as increased foetal loss, total resorption and abortion, were restricted to dosages exerting obvious toxic effects on the parent animal. Consequently they were not selective and perhaps even arose as a secondary consequence of the maternal effects. Total resorption of embryos in Nbs rabbits treated at 300 and 500 mg/kg could be partly connected with increase of uterine contraction due to the pharmacodynamic effect of thiamine, since Chemol (13) has stated that thiamine increased uterine contractions in experimental animals in vitro (guinia pig, cat, mouse, and rat) and in vivo (rabbit). This effect was more remarkable in pregnant rabbits.

The absence of selective teratogenic and other embryopathic effects observed in these studies, is in accord with the results of in vivo studies with mice, rats (see introduction), and rabbits (New Zealand white rabbits were treated with TTFD•HCl at daily doses of 50 and 200 mg/kg during the period of organogenesis) (14) and together constitute a volume of evidence counteracting the implications of the depression of cell division in in vitro studies (see introduction).
Further evidence that the in vivo studies offer a more relevant guide to possible effects in man is provided by the results of comparative metabolic, pharmacological and toxicological studies. Comparative metabolic studies of TTFD·HCl in the rat, rabbit and man showed that the metabolic fate of TTFD·HCl is essentially similar in its qualitative aspect (15). Further, all 5 final metabolites of TTFD side chain, methyl tetrahydrofurfuryl sulfoxide, methyl tetrahydrofurfuryl sulfone, d-methylsulfinyl-γ-valerolactone, d-methylsulfanyl-γ-valerolactone, and γ-hydroxy-d-methylsulfonyl valeric acid, have proved to be markedly less toxic than TTFD·HCl per se in mice and rats (16). In a qualitative aspect, the total B1 blood level in rabbits receiving 50 mg/kg (PO) (17) and 10 mg/rabbit (IV) (18) of TTFD·HCl attained a peak in one hour (ca. 470 µg/dl) and 10 minutes (ca. 2000 µg/dl) after dosing, respectively, and it was long lasting. Similarly, the peak of the total B1 blood level in man taken 300 mg (PO) (19) and 50 mg (IV) (20) was observed after 3 hours (114.4 µg/dl) and 30 minutes (200–240 µg/dl), respectively, and the blood level was long lasting. These results suggest that the status of blood level of TTFD·HCl following the administration is essentially similar between man and rabbit.

Markedly less toxicity of TTFD·HCl was presented in the subacute and chronic toxicity studies in the mouse, rat and dog (21–25), in the metabolic study of TTFD side chain (15, 26, 27) and in the pharmacological study of TTFD side chain (16, 28). Depression of the cell division by TTFD·HCl observed in vitro studies (see introduction) was not observed in vivo studies (29). Further, no inhibitory effects of TTFD·HCl on several SH-related enzyme systems in the rat was observed in the in vivo study (30). In the reproduction test including semi-generation test, untoward effects of TTFD·HCl were completely excluded in animals including offsprings which were treated (31). This evidence leads to the assumption that TTFD·HCl in vivo, if accumulated in considerable amounts intracellularly can be effectively metabolized into thiamine and less toxic metabolites cited above (32), consequently TTFD·HCl does not possess either selective embryotoxicity or teratogenicity.

SUMMARY

Pregnant rabbits (albino and a smaller albino colonies) and cynomolgus monkeys were given thiamine tetrahydrofurfuryl disulfide·HCl (TTFD·HCl) orally during the period of organogenesis at dose levels yielding 30, 300 and 500 mg/kg.

No significant increase in the incidence of foetal malformations was observed, even in groups treated with TTFD·HCl at dosages (300 and 500 mg/kg) exerting adverse maternal reaction, such as anorexia, reduced weight gain, or diarrhoea. Other embryo- or foeto-pathic effects, such as the increase of intrauterine mortality, foetal total resorption or abortion were restricted to dosages exerting adverse maternal reaction.

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