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EFFECTS OF CHRONIC MANGANESE (Mn_3O_4) EXPOSURE ON SELECTED REPRODUCTIVE PARAMETERS IN RATS

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Long-Evans rats were chronically exposed to dietary Mn_3O_4 beginning on d 1 of gestation and continuing through 224 d of age. Dietary concentrations of Mn, as Mn_3O_4, were 350, 1050, and 3500 ppm and were applied in either a normal Fe 240 ppm) or a low-Fe (20 ppm) basal diet. General toxic effects were apparent in young animals at a dietary dose of 3500 ppm Mn and were enhanced by concomitant Fe deficiency. Fertility was reduced in the group exposed to 3500 ppm Mn with a diet containing sufficient Fe. Male reproductive development was delayed by Mn treatment, as measured by testes weight, sperm count, and serum follicle-stimulating hormone and testosterone concentrations.

INTRODUCTION

Methylcyclopentadienyl manganese tricarbonyl (MMT) is used in fuels to improve combustion and to reduce smoke and particulate emissions, and its major combustion by-product is Mn_3O_4. Although the use of MMT in automotive fuels is currently prohibited by the Clean Air Act (Public Law 91-604, 1970), suspension of this ban during a shortage of unleaded fuel from June 5 to October 1, 1979, allowed up to 1/32 g MMT per gallon (Federal Register, 1979). This addition of MMT was allowed to prevent switching from unleaded to leaded fuel in vehicles with catalytic converters. Other sources of environmental Mn include ferromanganese alloy production, coal-fired power plants, and burning of residual and crude oil. These sources emit Mn to the environment primarily as manganese oxides (MnO, MnO_2, Mn_2O_3, and Mn_3O_4) (Ter Haar et al., 1975).

Mn is among the least toxic trace elements. Its turnover in the adult rat is directly related to its level in the diet, and tissue Mn concentrations remain relatively constant over a wide range of metabolic burdens of this metal.

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However, absorption of Mn by the preweanling rat (<21 d) is relatively high, resulting in higher toxicity. The LD50 (chronic) of Mn for preweanling rats has been reported as 35 mg/kg·d (as $MnCl_2$) (T. Ward, personal communication) and 225 mg/kg·d (as Mn_3O_4) (Rehnberg et al., 1980). In addition, tissue barriers to Mn accumulation in the young rat are not efficient, and tissue concentrations of Mn increase well beyond normal levels when Mn exposure is initiated before weaning (Rehnberg et al., 1980, 1981a; Ward, personal communication). The increased accumulation of Mn in the hypothalamus and pituitary during this period is of particular interest because of the potential toxic effects on reproductive development.

Mn and Fe share the same transport mechanism, and in Fe deficiency Mn absorption as well as Fe absorption is increased (Thompson et al., 1971; Thompson and Valberg, 1972). In adult rats the body compensates for changes in Mn absorption for the most part by increased Mn excretion. Elevated Mn tissue levels were observed during concurrent Mn exposure and Fe deficiency (Rehnberg et al., 1981b). Chronic Mn exposure of the young rat and the Fe-deficient rat resulted in increased accumulation of Mn in the brain and pituitary. This study was designed to evaluate the effects of chronic Mn_3O_4 exposure and concurrent Fe deficiency on reproductive development in the rat.

MATERIALS AND METHODS

Pregnant nulliparous Long-Evans rats (Blue Spruce Farms, Altmont, N.Y.) were received at our laboratory on d 1 of gestation. Animals were randomly assigned to one of eight treatment groups. Four groups received a basal diet containing 240 mg/kg Fe [as $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$][1], and four groups received a basal diet containing 20 mg/kg Fe. Both basal diets contained 50 mg/kg Mn as $MnSO_4$ and were supplemented with 0, 350, 1050, or 3500 mg/kg Mn as Mn_3O_4. They were prepared biweekly, stored at 10°C, and used within 2–4 wk. Food and water consumption was measured in F_1 animals periodically between 30 and 140 d. Dietary Fe content was determined by the supplier to be within 10% of the stated values. Mn and Fe concentrations in tap water were 0.05 ± 0.02 ppm and less than 0.5 ppm, respectively, as determined by flameless atomic absorption spectrophotometry. Mn_3O_4 was obtained from Alfa Products, Danvers, Mass., and was reported to be 98% pure. Purity was confirmed by atomic absorption spectrophotometry and particle size was determined by microscopy (Grainger Laboratories, Raleigh, N.C.) (Table 1).

Mn treatment began on d 2 of pregnancy and continued for the duration of the study. Animals were allowed food and water *ad libitum*

[1] Base diets TD-76476 (240 mg/kg Fe) and TD-76475 (20 mg/kg Fe); Teklad Test Diets, Madison, Wis.

TABLE 1. Particle Size and Distribution of Mn_3O_4

Particle size (μm)	Percent
0.3 or less	3.0
0.31–0.60	19.5
0.61–0.90	13.0
0.91–1.20	20.5
1.21–1.50	9.5
1.51–1.80	12.5
1.81–2.10	5.5
2.11–2.40	7.0
2.41–2.70	1.5
2.71–3.00	3.5
3.00 or greater	4.5

and were maintained on a 12:12 light-dark cycle starting at 1000 h. On d 1 or 2 postpartum, litters were reduced to 3 males and 3 females each (fostering when necessary) and weaned at 24 d of age. Five to eight nonlittermate males and/or females from each dose group were randomly selected, weighed, and killed at 24, 40, 60, 100, or 224 d of age.

Testes from all age groups were removed, trimmed, and weighed. At 60, 100, and 224 d of age the epididymides were removed, trimmed, and minced in 2 ml normal saline, and then 8 ml 10% buffered Formalin was added. Aliquots of sperm from the minced epididymides were diluted with 10% buffered Formalin and counted with a Coulter electronic counter (model ZBI, Coulter Electronics, Hialeah, Fla.). Precision was evaluated by counting 10% of the samples with a hemacytometer. Serum was prepared from cardiac blood samples obtained between 0945 and 1015 h from animals 40, 60, and 100 d of age.

At 90–100 d of age, nonlittermate males and females from each dose group were caged (1 male with 2 females) for 2 wk. Deliveries by term females (females having delivered 1–4 pups) were completed by cesarean section and pups were removed, cleaned, dried, and weighed. Uterine implantation sites were counted, ovaries removed, and corpora lutea counted.

Serum testosterone was assayed by the radioimmunoassay (RIA) method of Furuyama et al. (1970) as modified by Chen et al. (1971). Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were assayed by the RIA procedure outlined by the National Institutes of Arthritis, Metabolism, and Digestive Diseases (NIAMD) and Rat Pituitary Hormone Distribution Program (Benson et al., 1964; Midgley, 1966, 1967; Faiman and Ryan, 1967; Greenwood et al., 1963; Odell et al., 1967), using NIAMD rat FSH-RP-1 and NIAMD rat LH-RP-1 as standards.

Data were analyzed by two-way analysis of variance (ANOVA),

analysis of covariance, Dunnett's multiple comparison procedure, and chi-square analysis when appropriate with a computer-based statistical analysis system (Helwig and Council, 1979).

RESULTS

The average Mn_3O_4 particle diameter was 1.02 μm, and 78% of the particles were less than 1.8 μm in diameter (Table 1). Mortality of all animals (males and females) on the low-Fe diet with 3500 mg/kg Mn exceeded 90% by d 50. Evaluations of this treatment group were not continued beyond d 40. No mortality occurred in any other treatment group, indicating an LD50 (chronic) of more than 3500 mg/kg for rats receiving a normal Fe diet and of 1050–3500 mg/kg for rats receiving a low-Fe diet.

No significant differences in food consumption (and therefore Mn intake) per kilogram of body weight were noted (Carter et al., 1980). Body weight data from males and females are presented in Tables 2 and 3,

TABLE 2. Body Weights of Males during Chronic Exposure to Mn_3O_4

Dietary Mn as Mn_3O_4 (ppm)	Body weight (g) at age				
	24 d	40 d	60 d	100 d	224 d
Normal Fe diet (240 ppm)					
0 (control)	(5)[a] 80 ± 8[b]	(6) 163 ± 9	(6) 296 ± 17	(6) 462 ± 39	(8) 684 ± 90
350	(6) 70 ± 9	(6) 163 ± 15	(6) 308 ± 20	(6) 424 ± 57	(7) 711 ± 54
1050	(6) 71 ± 7	(6) 151 ± 20	(6) 299 ± 15	(6) 462 ± 47	(8) 640 ± 67
3500	(6) 68 ± 9	(6) 146 ± 18	(6) 320 ± 25	(6) 463 ± 28	(8) 663 ± 76
Low-Fe diet (20 ppm)					
0	(6) 67 ± 16	(7) 156 ± 19	(6) 264 ± 22[c]	(6) 418 ± 19	(7) 688 ± 68
350	(6) 69 ± 8	(6) 138 ± 13[c]	(6) 247 ± 28[c]	(6) 413 ± 46	(8) 613 ± 61
1050	(6) 53 ± 15[c]	(6) 103 ± 31[c]	(6) 236 ± 31[c]	(6) 357 ± 46[c]	(8) 660 ± 96
3500	–	(5) 58 ± 17[c]	–	–	–

[a]Sample number.
[b]Mean ± SD.
[c]Means are significantly different from control values at $p < 0.05$ (ANOVA and Dunnett's multiple comparison test).

TABLE 3. Body Weights of Females during Chronic Exposure to Mn_3O_4

Dietary Mn as Mn_3O_4 (ppm)	Weight (g) at age			
	40 d	60 d	100 d	224 d
Normal Fe diet (240 ppm)				
0 (control)	(5)[a] 134 ± 10[b]	(5) 220 ± 13	(6) 280 ± 24	(6) 355 ± 30
350	(6) 132 ± 19	(6) 219 ± 20	(6) 278 ± 24	(8) 320 ± 40
1050	(6) 146 ± 4	(6) 216 ± 15	(6) 285 ± 14	(8) 331 ± 30
3550	(7) 123 ± 18	(6) 205 ± 16	(6) 253 ± 14	(8) 365 ± 73
Low-Fe diet (20 ppm)				
0	(6) 133 ± 17	(6) 196 ± 21	(6) 313 ± 26	(7) 367 ± 54
350	(6) 111 ± 31	(6) 183 ± 13[c]	(6) 286 ± 37	(8) 340 ± 36
1050	(6) 120 ± 18	(5) 187 ± 20[c]	(6) 269 ± 41	–
3550	(6) 42 ± 11[c]	–	–	–

[a]Sample number.
[b]Mean ± SD.
[c]Means are significantly different from control values at $p < 0.05$ (ANOVA and Dunnett's multiple comparison test).

respectively. There was no effect of Mn on male or female body weight at any age in animals receiving a normal Fe diet. Body weights of males receiving Mn in a low-Fe diet were significantly depressed ($p < 0.05$) through 100 d, while females' body weights were depressed only through 60 d.

Ovary weight data were analyzed by ANOVA and analysis of covariance with body weight as a covariable, and no significant differences were detected. Table 4 shows data on the reproductive parameters of rats bred at 90–100 d. Statistical analysis of the data did not identify any significant Mn- or Fe-related effects on the parameters examined, except for fertility. There was a reduction in fertility in the group receiving 3500 mg/kg Mn (chi-square analysis, $p < 0.05$).

Testes weight data (Table 5) were analyzed by ANOVA and analysis of covariance with body weight as the covariable. ANOVA showed significant Fe effects at 40, 60, and 100 d of age. Mn-related effects were seen only in the Fe-deficient group at 40 and 100 d of age. Analysis of covariance, with body weight as the covariable, showed no significant testes weight differences.

TABLE 4. Selected Reproductive Parameters for Females Chronically Exposed to Mn_3O_4

Parameter	Dietary Mn as Mn_3O_4 (ppm)			
	0	350	1050	3500
Percent pregnant	(43)[a]	(45)	(47)	(24)
	84	84	79	63[b]
Litter size (average per litter)	(20)[c]	(19)	(20)	(10)
	10.5	10.4	9.8	10.6
Ovulations (average per dam)	(20)[c]	(19)	(20)	(10)
	15.6	15.2	14.5	15.0
Resorptions (average per litter)	(20)[c]	(19)	(20)	(10)
	2.2	1.6	2.6	1.6
Preimplantation deaths	(20)[c]	(19)	(20)	(10)
(average per litter)	2.9	3.2	2.1	2.8
Mean F_2 fetal weights (g)	(20)[c]	(19)	(20)	(10)
Males	5.7	6.0	6.0	6.1
Females	5.5	5.6	5.6	5.8

[a]Number of females bred.
[b]Significantly different ($p < 0.05$) from control (chi-square analysis).
[c]Number of litters.

Serum hormone concentrations and epididymal sperm counts from males 60 and 100 d of age are presented in Tables 6–9. No treatment effects were seen with 3500 ppm Mn at 40 d of age in serum LH, FSH, or testosterone concentrations. Combined values for controls and Mn-treated males were 45 ± 20, 552 ± 226, and 1.35 ± 0.65 ng/ml (mean ± SD), respectively. Of particular interest is the Mn dose-related decrease in serum testosterone concentration without a concomitant increase in serum LH concentration. The normal decline in serum FSH concentration between 60 and 100 d is prevented by Mn treatment in both Fe groups. Epididymal sperm counts were depressed in the Fe-deficient Mn-treated group only at 100 d. Even the group treated with 3500 ppm Mn in a normal Fe diet was unaffected ($119 \pm 28 \times 10^6$). In addition, when epididymal sperm counts were evaluated at 224 d no differences were noted (overall mean, $134 \pm 48 \times 10^6$).

DISCUSSION

Under normal dietary regimens, the testes, pituitary, and hypothalamus accumulate very little Mn. However, with chronic oral Mn_3O_4 administration during the preweanling period, the Mn concentration in the hypothalamus and pituitary exceeds that of the liver, the primary soft tissue target organ in the adult, by weaning (21–24 d postpartum) (Rehnberg et

al., 1981a). In addition, Mn accumulated in these tissues during the preweaning period is retained longer than that accumulated during the postweaning period (Rehnberg et al., 1981a, 1981b; Cahill et al., 1980). Mn accumulation in the testes under the same preweanling exposure regimens was minimal. This pattern of Mn accumulation in the hypothalamus and pituitary would be consistent with the altered pattern of release of gonadotropic hormones from the anterior pituitary and testosterone from the testes seen in this study.

Daily oral administration of Mn_3O_4 from d 1 postpartum demonstrated and LD50 (21-d chronic) of 225 mg/kg·d Mn (Rehnberg et al., 1980). In this study, animals did not begin feeding on the Mn-treated food until d 14–15 postpartum. Food consumption ranged from 40 g/kg·d in adults rats (>100 d) to 80 g/kg·d in young rats (≥50 d) (Carter et al., 1980). From these food consumption figures, Mn consumption for the dose groups receiving 350, 1050, and 3500 mg/kg Mn was 16–32, 44–88, and 158–316 mg/kg·d, respectively. The mortality in the 3500 mg/kg dose group in this study indicates an LD50 (chronic) of 88–316 mg/kg·d in rats

TABLE 5. Testes Weights during Chronic Exposure to Mn_3O_4

Dietary Mn as Mn_3O_4 (ppm)	Weight (g) at age				
	24 d	40 d	60 d	100 d	224 d
Normal Fe diet (240 ppm)					
0 (control)	(6)[a] 0.36 ± 0.02[b]	(6) 1.70 ± 0.14	(6) 3.15 ± 0.71	(6) 3.32 ± 0.30	(8) 3.91 ± 0.90
350	(6) 0.33 ± 0.05	(6) 1.67 ± 0.24	(6) 3.14 ± 0.40	(6) 3.12 ± 0.46	(7) 3.96 ± 0.89
1050	(6) 0.36 ± 0.05	(6) 1.54 ± 0.17	(6) 2.85 ± 0.25	(6) 3.40 ± 0.26	(8) 3.26 ± 0.82
3500	(6) 0.37 ± 0.04	(5) 1.67 ± 0.15	(6) 2.97 ± 0.62	(6) 3.56 ± 0.34	(8) 3.06 ± 0.95
Low-Fe diet (20 ppm)					
0	(6) 0.32 ± 0.08	(6) 1.49 ± 0.17	(6) 2.62 ± 0.46	(6) 3.17 ± 0.19	(7) 3.74 ± 0.46
350	(6) 0.37 ± 0.02	(6) 1.47 ± 0.23	(6) 2.75 ± 0.18	(6) 2.94 ± 0.15	(8) 3.73 ± 0.27
1050	(6) 0.29 ± 0.10	(6) 1.11 ± 0.38[c]	(6) 2.64 ± 0.42	(6) 2.41 ± 0.77[c]	(8) 3.39 ±0.38
3500	–	(5) 0.60 ± 0.20[c]	–	–	–

[a]Sample number.
[b]Mean ± SD.
[c]Means are significantly different from control values at $p < 0.05$ (ANOVA and Dunnett's multiple comparison test).

TABLE 6. Serum Testosterone during Chronic Exposure to Mn_3O_4[a]

Dietary Mn as Mn_3O_4[c] (ppm)	Serum testosterone (ng/ml) at age[b]		
	60 d	100 d	Pooled over age
Normal dietary Fe (240 ppm)[d,e]			
0	2.21 ± 1.22[f]	5.28 ± 3.30	3.61
350	2.58 ± 1.62	2.65 ± 1.30	2.61
1050	2.38 ± 1.61	1.72 ± 0.69	2.05
Pooled over dose	2.39	3.10	2.73[d]
Low dietary Fe (20 ppm)			
0	2.60 ± 0.79	5.79 ± 3.85	4.19
350	2.33 ± 1.35	5.10 ± 2.65	3.72
1050	1.41 ± 0.90	2.18 ± 1.04	1.80
Pooled over dose	2.11	4.36	3.24
Pooled over dietary Fe			
0	2.41	5.56	3.91[c]
350	2.46	3.88	3.17
1050	1.90	1.95	1.92
Pooled over dose and Fe	2.25[b]	3.74	

[a]A log transformation was done before statistical analysis; $n = 6$ per group.
[b]Age main effect was significant ($p < 0.003$).
[c]Dose main effect was significant ($p < 0.0003$).
[d]Iron main effect was not significant ($p > 0.50$).
[e]Age X dose, age X Fe, and dose X Fe interactions were not significant ($p > 0.10$).
[f]Mean ± SD.

TABLE 7. Serum LH during Chronic Exposure to Mn_3O_4[a]

Dietary Mn as Mn_3O_4[c] (ppm)	Serum LH (ng/ml) at age[b]		
	60 d	100 d	Pooled over age
Normal dietary Fe (240 ppm)[d,e]			
0	93 ± 27[f]	128 ± 89	109
350	104 ± 43	99 ± 44	101
1050	82 ± 12	87 ± 15	84
Pooled over dose	93	103	98[d]
Low dietary Fe (20 ppm)			
0	82 ± 36	88 ± 5	85
350	78 ± 12	74 ± 51	76
1050	67 ± 13	88 ± 4	78
Pooled over dose	76	84	80
Pooled over dietary Fe[e]			
0	88	106	97[c]
350	91	87	89
1050	74	88	81
Pooled over dose and Fe	84[b]	93	

[a]A log transformation was done before statistical analysis; $n = 6$ per group.
[b]Age main effect was not significant ($p > 0.5$).
[c]Dose main effect was not significant ($p > 0.4$).
[d]Fe main effect was significant ($p < 0.03$).
[e]Age X dose, age X Fe, and dose X Fe interactions were not significant ($p > 0.20$).
[f]Mean ± SD.

TABLE 8. Serum FSH during Chronic Exposure to Mn_3O_4[a]

Dietary Mn as Mn_3O_4[c] (ppm)	Serum LH (ng/ml) at age[b]		
	60 d	100 d	Pooled over age
Normal dietary Fe (240 ppm)[d,e,f]			
0	555 ± 131	347 ± 57[f]	460
350	539 ± 140	368 ± 175	453
1050	570 ± 112	525 ± 96	548
Pooled over dose	555[f]	417	488[d]
Low dietary Fe (20 ppm)[g]			
0	606 ± 269	409 ± 129	507
350	508 ± 105	590 ± 88	549
1050	464 ± 51	509 ± 124	487
Pooled over dose	526	503	514
Pooled over dietary Fe			
0	580	380	485[c]
350	523	479	501
1050	517	517	517
Pooled over dose and Fe	540[b]	461	

[a]A log transformation was done before statistical analysis; $n = 6$ per group.
[b]Age main effect was significant ($p < 0.01$).
[c]Dose main effect was not significant ($p > 0.30$).
[d]Fe main effect was not significant ($p > 0.30$).
[e]Age × dose and age × Fe interactions were significant ($p < 0.05$). Dose × Fe interaction was not significant ($p > 0.10$).
[f]Age ($p < 0.001$) effects and dose ($p < 0.02$) effects (at 100 d) were significant in the normal Fe group.
[g]No significant age ($p > 0.50$) or dose ($p > 0.50$) effects were seen in the low-Fe group.

TABLE 9. Epididymal Sperm during Chronic Exposure to Mn_3O_4[a]

Dietary Mn as Mn_3O_4[c] (ppm)	Sperm ($\times 10^{-6}$) at age[b]		
	60 d	100 d	Pooled over age
Normal dietary Fe (240 ppm)[d,e]			
0	56 ± 15[f]	132 ± 85	90
350	44 ± 6	109 ± 42	77
1050	57 ± 12	110 ± 9	84
Pooled over dose	53	116	84[d]
Low dietary Fe (20 ppm)			
0	41 ± 8	103 ± 34[g]	72
350	39 ± 15	86 ± 40	62
1050	34 ± 8	59 ± 43	47
Pooled over dose	38	83	60
Pooled over dietary Fe			
0	48	116	81[c]
350	42	97	69
1050	46	85	66
Pooled over dose and Fe	45[b]	99	

[a]A log transformation was done before statistical analysis, $n = 6$ per group.
[b]Age main effect was significant ($p < 0.0001$).
[c]Dose main effect was not significant ($0.05 < p < 0.10$).
[d]Fe main effect was significant ($p < 0.0001$).
[e]Age × dose and age × Fe interactions were not significant ($p > 0.20$). Dose × Fe interaction was significant ($p < 0.050$).
[f]Mean ± SD.
[g]Dose effect was significant ($p < 0.01$) in the low-Fe group at 100 d.

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receiving an Fe-deficient diet. This is consistent with the LD50 (21-d chronic) of 225 mg/kg·d reported by Rehnberg et al. (1980). It was also noted by Rehnberg et al. (1980) that the normal preweanling rat is essentially anemic, with liver Fe concentrations of 30–50 μg/g tissue, compared to 150–160 μg/g for adult males.

In a study of CD-1 mice chronically exposed to Mn_3O_4 (1050 mg/kg Mn in the diet), Gray and Laskey (1980) reported retardation of male sexual development as evidenced by reduced testes, seminal vesicle, and preputial weights. In addition, Gray et al. (1978) showed a reduction in spontaneous behavior ($p < 0.01$) in postpubertal (45–100 d) male and female rats from this study in the 1050 mg/kg Mn dose group with a normal Fe supplement.

In this study, significant Mn effects on the male reproductive system were seen through 100 d in rats dosed with 1050 mg/kg Mn. None of the effects seen were severe enough to alter functional reproduction. The onset of male sexual maturation was apparently unimpaired, with minimal effects seen in the measured reproductive parameters at 40 and 60 d. By 100 d, however, the reproductive effect in males is quite evident with chronic Mn exposure in both Fe groups.

Serum concentrations of LH, FSH, and testosterone and epididymal sperm counts from the 60- and 100-d control animals receiving a normal Fe diet were used to predict the reproductive age of all animals by discriminant analysis, an extension of the multivariant analysis of variance procedure (Li, 1964); Kleinbaum and Kupper, 1978; Helwig and Council, 1979). All animals were classifed with the assumption that they had an equal probability of belonging to either the 60- or 100-d-old group. All of the 60-d-old animals were classified correctly. When normal and low-Fe 100-d-old animals were evaluated, 2 of 12 controls, 7 of 12 animals receiving 350 ppm Mn, and 12 of the 12 animals receiving 1050 ppm Mn were classified as 60 d old. This indicates a considerable Mn-related maturational delay.

Studies are continuing to determine whether Mn acts as a general toxin on the reproductive axis or at a specific site in the axis.

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