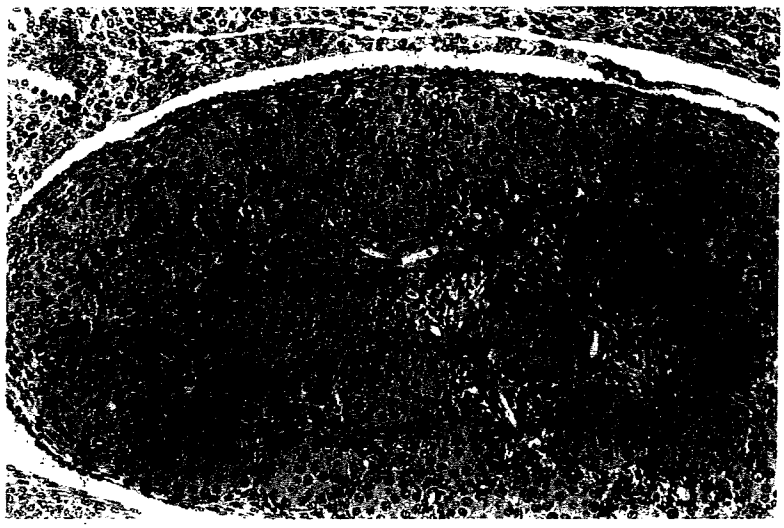

Photo 1-a. Transverse section of a fetus from the control group on G16 showing the genital ridge. HE stain, ×160.

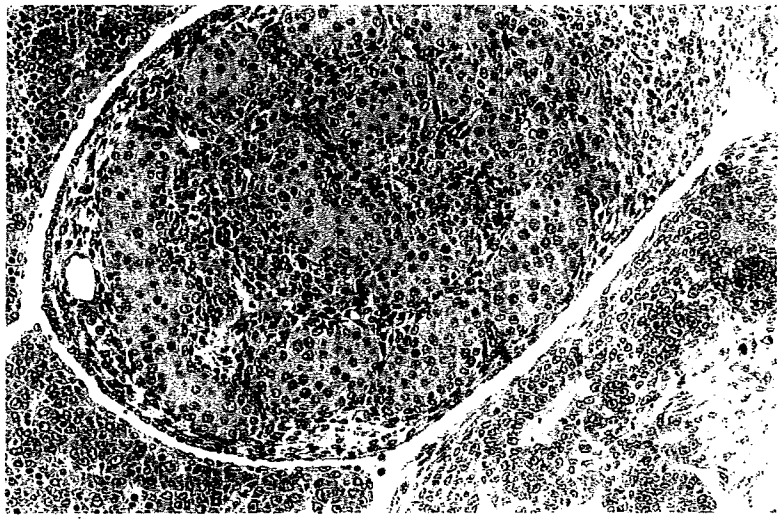

Photo 1-b. Transverse section of a fetus from the 1000 mg/kg DEHP group on G16 showing no abnormality in the genital ridge. HE stain, ×160.

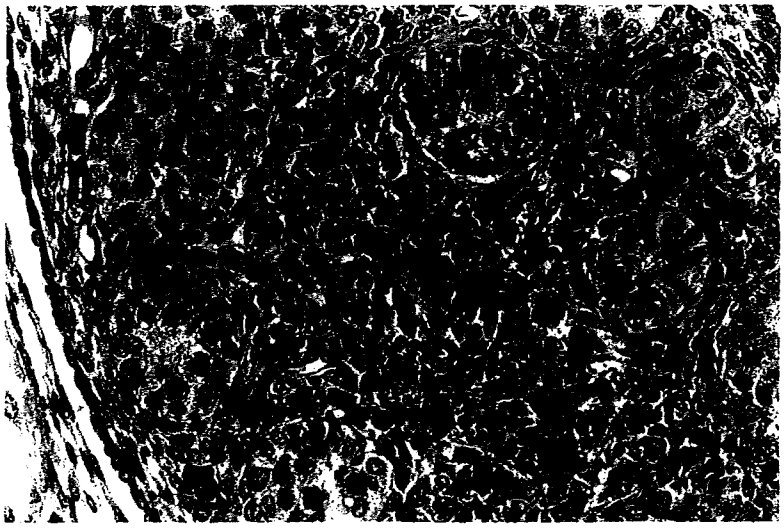

Photo 1-c. Transverse section of a fetus from the 1000 mg/kg DEHP group on G16 showing the genital ridge. Many germ cells are degenerated and densely stained. HE stain, ×310.

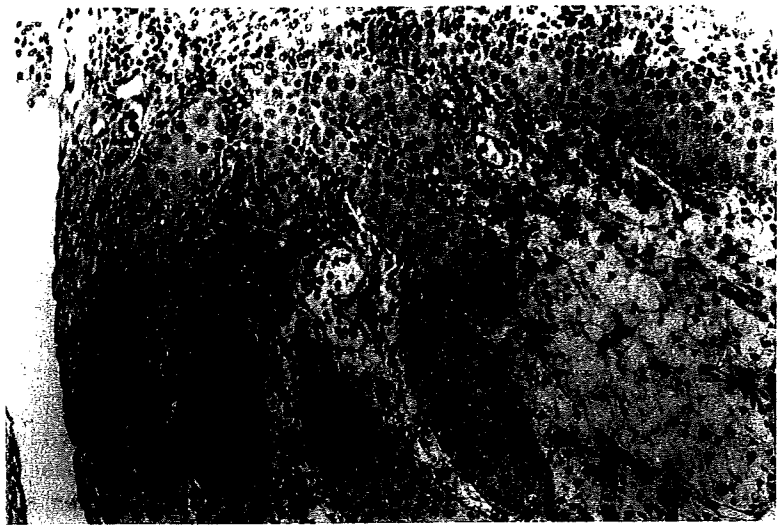

Photo 2-a. Testis of a G18 rat fetus from the control group. HE stain, ×160.

Photo 2-b. Testis of a G18 fetus from a rat treated with 500 mg/kg of DEHP showing hyperplasia of interstitial cells. HE stain, ×160.

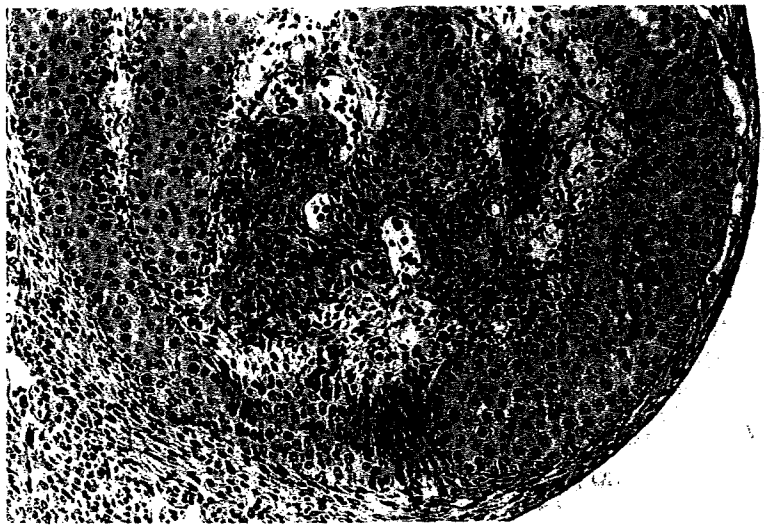

Photo 2-c. Testis of a G18 fetus from a rat treated with 1000 mg/kg DEHP showing hyperplasia of interstitial cells. HE stain, ×160.

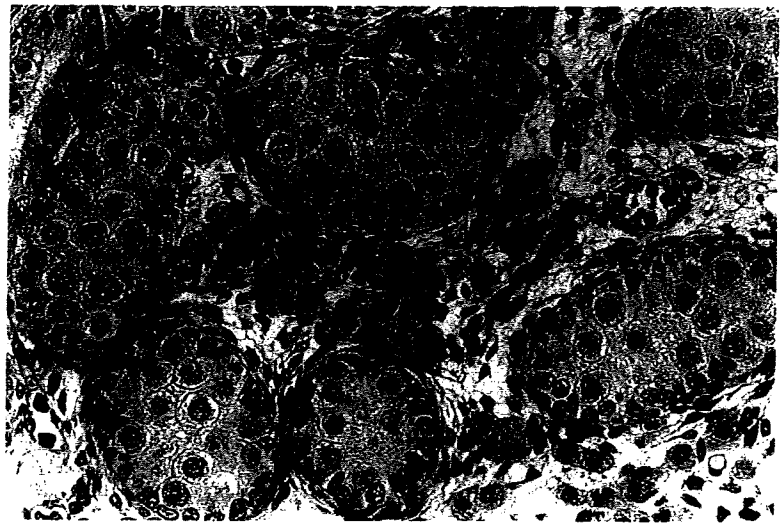

Photo 3-a. Testis of a G20 fetus from the control group showing the seminiferous cords and interstitial cells. HE stain, ×310.

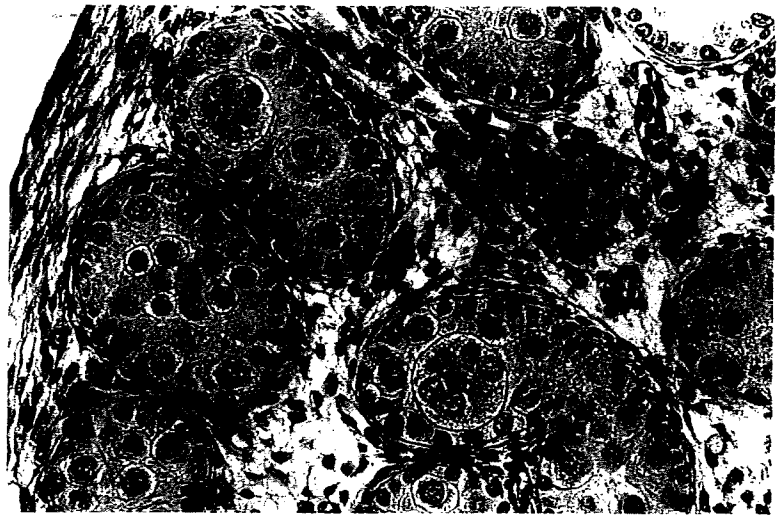

Photo 3-b. Testis of a G20 fetus from the group treated with 500 mg/kg of DEHP showing multinucleated germ cells in seminiferous cords. HE stain, ×310.

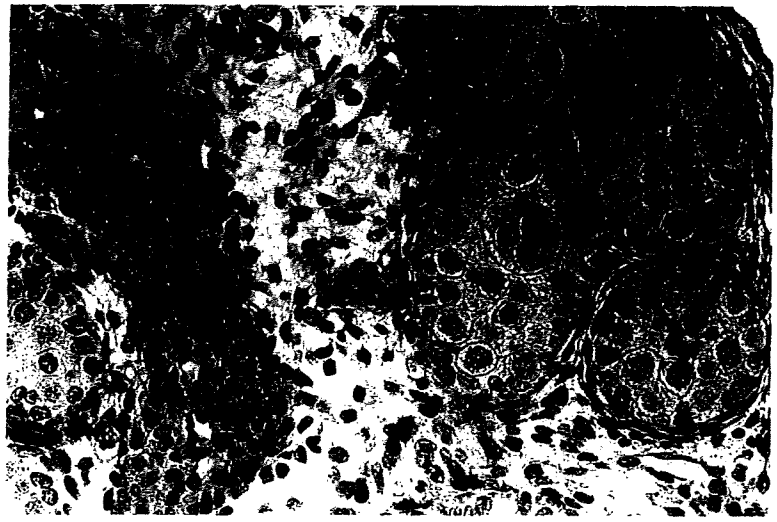

Photo 3-c. Testis of a G20 fetus from the group treated with 1000 mg/kg of DEHP showing multinucleated germ cells in seminiferous cords, and hyperplastic smaller-sized interstitial cells. HE stain, ×310.

5a, 5b). In their epididymides, atrophy was found in all of the animals and cell debris in the epididymal lumen was also found (Photos 6a, 6b).

Effects of the lower doses of DEHP on testicular development were examined in Experiment 2. Table 6 summarizes histopathological findings of fetal testes on G20 and testes of offspring at 5 and 10 weeks of age in Experiment 2. Multinucleated germ cells were found in the fetal testes of all the groups exposed to DEHP, although its incidence was very low in the 125 mg/kg group. In the groups exposed to 250 mg/kg and 500 mg/kg of DEHP, partly thickened germinal cords due to aggregation of increased number of germ cells and hyperplasia of the interstitial cells were observed. Degenerated germ cells and apoptosis were observed in a few animals in the group exposed to 500 mg/kg of DEHP. These findings are comparable to those in DEHP-exposed testes at the same dose in Experiment 1.

In contrast to the findings of the fetal testes, no abnormalities were found in testes of the offspring at 5 and 10 weeks of age in any group in histopathological examination. Furthermore, the seminiferous cycles in the testis determined at 5 weeks of age were comparable between control and DEHP-exposed groups (Table 7).

Electron microscopic findings of fetuses

Electron microscopic examination of fetal testes was performed in Experiment 1. In the fetal testis of the groups exposed to DEHP at 500 and 1000 mg/kg, degenerated germ cells were found in the testicular cord on G16 (Photo 7), and smaller-sized interstitial cells containing fewer lipid droplets were noted on G18 (Photo 8a). These changes of the interstitial cells became more obvious on G20 (Photo 8b).

In the fetal testis from the group exposed to EE at 0.5 mg/kg, degeneration of germ cells was found only on G14. No abnormalities such as those observed with DEHP treatment were found on G16, 18 and 20. Slightly swollen mitochondria and hyperplastic smooth endoplasmic reticulum were noted in interstitial cells on G18 and 20. Furthermore, degeneration of interstitial cells surrounded by neutrophils infiltration were observed on G20.

In examination of offspring at 5 and 10 weeks after birth in Experiment 2, ultrastructural changes were not observed in the testis and epididymides of any

Table 5. Histopathological findings in the testis and epididymis of offspring exposed to DEHP during gestational days 7-18 (Experiment 1) 7 weeks after birth.

Group	DEHP 500 mg/kg (6)					DEHP 1000 mg/kg (12)				
Grade	−	±	+	++	+++	−	±	+	++	+++
Testis										
Atrophy of seminiferous tubules	6	0	0	0	0	2	2	4	3	1
Multinucleated giant cells	5	1	0	0	0	0	3	5	4	0
Dilatation of seminiferous tubules	6	0	0	0	0	2	0	4	6	0
Dilatation of rete testis	6	0	0	0	0	8	2	1	1	0
Hyperplasia of interstitial cells	6	0	0	0	0	10	1	1	0	0
Necrosis	6	0	0	0	0	11	0	0	0	1
Mineralization	6	0	0	0	0	10	0	1	1	0
Foreign body giant cells	6	0	0	0	0	10	0	1	1	0
Focal loss of seminiferous tubules	6	0	0	0	0	11	0	1	0	0
Malformation of seminiferous tubules	6	0	0	0	0	11	0	1	0	0
Epididymis										
Atrophy	6	0	0	0	0	0	1	2	2	7
Cell debris in lumens	0	0	6	0	0	3	3	6	0	0
Dilatation of lumens	6	0	0	0	0	8	0	3	1	0
Infiltration of lymphocytes	6	0	0	0	0	8	2	2	0	0
Granuloma	6	0	0	0	0	11	0	0	1	0

Figures in parentheses indicate number of offspring examined.
− : not observed, ± : very slight, + : slight, ++ : moderate, +++ : severe.

Photo 4-a. Testis of a 7-week-old rat treated with 500 mg/kg of DEHP *in utero* showing no abnormalities in the seminiferous tubules and interstitial cells. HE stain, ×310.

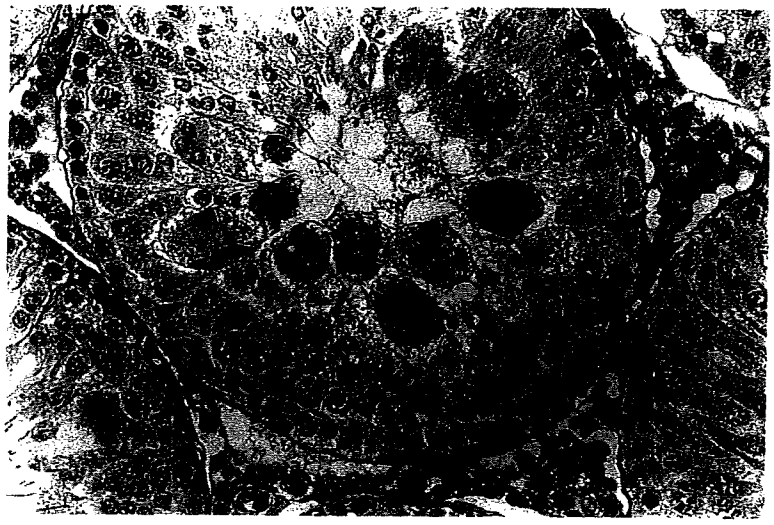

Photo 4-b. Testis of a rat of the same group as Photo 4-a, showing multinucleated giant cells in a seminiferous tubule. HE stain, ×310.

Photo 4-c. Testis of a 7-week-old rat treated with 1000 mg/kg of DEHP *in utero*, showing atrophy of seminiferous tubule epithelia with multinucleated giant cell in the lumen, and also hyperplasia of interstitial cells. HE stain, ×310.

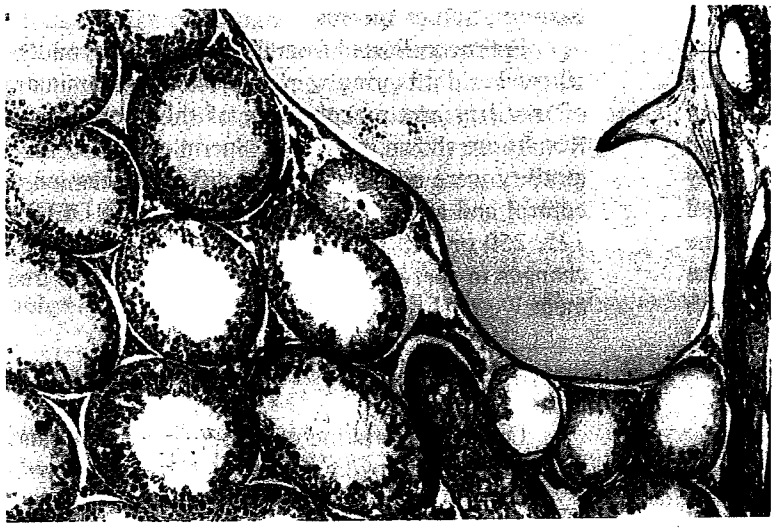

Photo 5-a. Testis of a 7-week-old rat treated with 1000 mg/kg of DEHP *in utero*, showing dilatation of seminiferous tubules and of rete testis. HE stain, ×80.

Photo 5-b. Testis from a 7-week-old rat treated with 1000 mg/kg of DEHP *in utero*, showing branching of atrophic seminiferous tubules. HE stain, ×80.

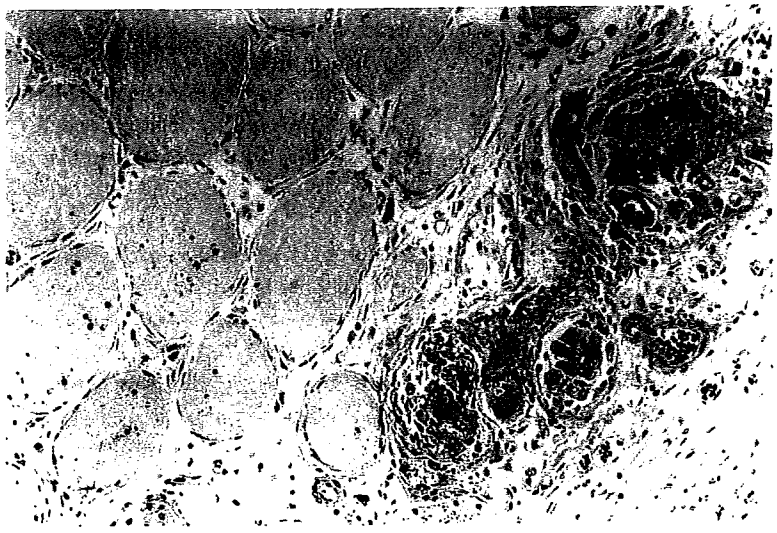

Photo 5-c. Testis of a 7-week-old rat treated with 1000 mg/kg of DEHP *in utero*, showing extensive necrosis and foreign body giant cells. HE stain, ×160.

of the offspring in any groups (control and 500 mg/kg DEHP groups).

Expression of androgen receptors

Immunohistochemical staining revealed an increase of androgen receptor-positive cells, namely hyperplasia of Leidig cells, in the interstitium of fetal testes at G20 in the 500 mg/kg group (Photo 9). In the offspring at 5 and at 10 weeks after birth, however, the expression of androgen receptors observed in Sertoli cells, myoid cells and interstitial cells was not different among the control and DEHP treated groups (data not shown).

Examination of sperms

Sperms collected from the cauda epididymidis of 10-week old offspring were subjected to examination of motility and morphology of the spermatozoa. Results are shown in Table 8. Sperm count and sperm motility were not significantly different between the control and any of the groups treated with DEHP at 125, 250 or 500 mg/kg. There were no remarkable changes in spermatogenic parameters related to treatment.

DISCUSSION

Oral administration of DEHP to pregnant rats at doses up to 1000 mg/kg from G7 to G18, which corre-

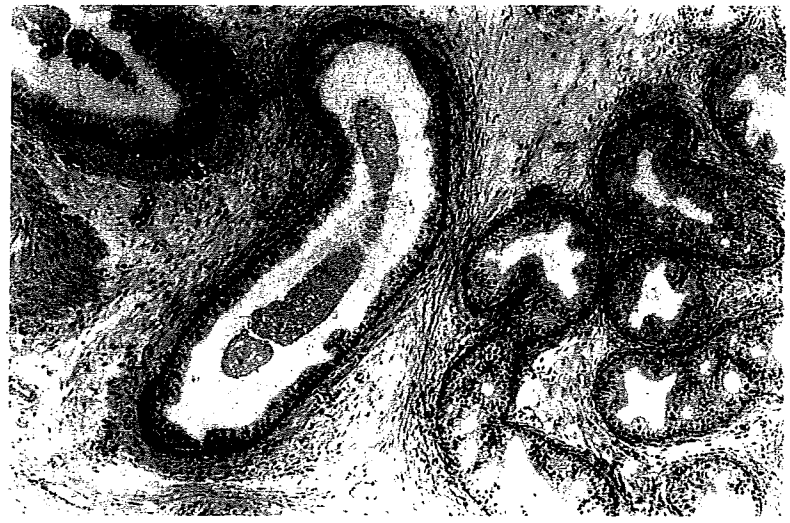

Photo 6-a. Epididymis of a 7-week old rat treated with 1000 mg/kg of DEHP *in utero*, showing atrophy of epididymal ducts and cell debris in the lumen. HE stain, ×80.

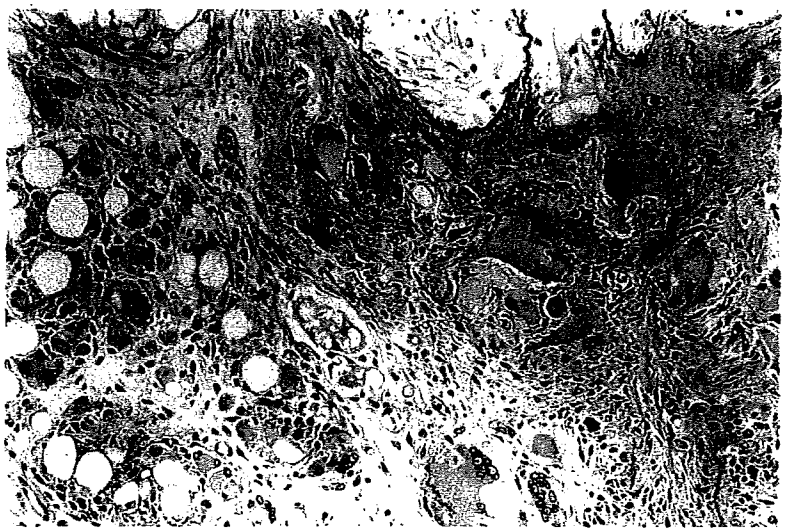

Photo 6-b. Granuloma formed in the epididymis of a 7-week-old rat treated with 1000 mg/kg of DEHP *in utero*, accompanied by numerous foreign body giant cells and fibrosis. HE stain, ×160.

sponded to the organogenetic period of a rat fetus, induced fetal damage such as increase in fetal mortality, inhibition of fetal weight gain, and some malformations in the highest dose. Histopathological studies revealed degeneration of germ cells and hyperplasia of interstitial cells in the fetal testis in the groups treated with DEHP at doses of 500 mg/kg and above. Similar changes were also observed in slight degree in the 250 mg/kg group but not in the 125 mg/kg group. Electron microscopic examination of these testes of affected groups revealed smaller-sized interstitial cells in which lipid droplets were depleted. Testicular toxicity of a phthalate ester by *in utero* exposure in rats have been described by Mylchreest *et al.* (2000) using di-(*n*-

Table 6. Histopathological findings of testes of offspring exposed to di-(2-ethylhexyl) phthalate (DEHP) during gestational days 7-18 (Experiment 2).

Group	DEHP 0 mg/kg [a]					DEHP 125 mg/kg					DEHP 250 mg/kg					DEHP 500 mg/kg				
Grade	−	±	+	++	+++	−	±	+	++	+++	−	±	+	++	+++	−	±	+	++	+++
Gestational day 20	(15)					(21)					(19)					(28)				
Multinucleated germ cells	15	0	0	0	0	16	5	0	0	0	4	15	0	0	0	2	25	1	0	0
Increase of germ cells in a cord	15	0	0	0	0	21	0	0	0	0	16	3	0	0	0	1	21	6	0	0
Hyperplasia of interstitial cells	15	0	0	0	0	21	0	0	0	0	6	12	1	0	0	6	5	17	0	0
Degeneration of germ cells	15	0	0	0	0	21	0	0	0	0	19	0	0	0	0	26	2	0	0	0
Apoptosis of germ cells	15	0	0	0	0	21	0	0	0	0	19	0	0	0	0	27	1	0	0	0
5 weeks after birth	(4)					(4)					(4)					(4)				
Abnormalities	4	0	0	0	0	4	0	0	0	0	4	0	0	0	0	4	0	0	0	0
10 weeks after birth	(4)					(4)					(4)					(4)				
Abnormalities	4	0	0	0	0	4	0	0	0	0	4	0	0	0	0	4	0	0	0	0

[a] Vehicle control (corn oil, 5 mL/kg). Figures in parentheses indicate number of fetuses or offspring examined.
− : not observed, ± : very slight, + : slight, ++ : moderate, +++ : severe.

Table 7. Morphometric analysis of spermatogenesis of the offspring exposed to 500 mg/kg of di-(2-ethylhexyl)phthalate (DEHP) during gestational days 7-18 5 weeks after birth (Experiment 2).

	DEHP 0 mg/kg [a]	DEHP 500 mg/kg
(Number of offspring examined)	(4)	(4)
Group 1 (Stage I~VI)		
Count of germ cells in a seminif. tubule	1098.5 ± 43.4	1150.8 ± 110.9
Count of Sertoli cells in a seminif. tubule	133.8 ± 7.9	130.8 ± 4.6
Germ cells/Sertoli cells	8.2 ± 0.7	8.8 ± 1.1
Group 2 (Stage VII~VIII)		
Count of germ cells in a seminif. tubule	1026.5 ± 84.3	1039.3 ± 24.4
Count of Sertoli cells in a seminif. tubule	137.0 ± 7.4	120.5 ± 9.0
Germ cells/Sertoli cells	7.5 ± 0.9	8.7 ± 0.7
Group 3 (Stage IX~XI)		
Count of germ cells in a seminif. tubule	933.8 ± 66.5	938.3 ± 20.9
Count of Sertoli cells in a seminif. tubule	135.3 ± 3.0	125.0 ± 8.2
Germ cells/Sertoli cells	6.9 ± 0.6	7.5 ± 0.4
Group 4 (Stage XII~XIV)		
Count of germ cells in a seminif. tubule	768.5 ± 28.9	738.8 ± 62.9
Count of Sertoli cells in a seminif. tubule	130.8 ± 7.0	127.0 ± 9.7
Germ cells/Sertoli cells	5.9 ± 0.5	5.8 ± 0.2

Values represent mean ± S.D.
[a] Vehicle control (corn oil, 5 mL/kg).

'butyl)phthalate (DBP). They made oral administration of DBP at doses of 0.5, 5, 50, 100 and 500 mg/kg to pregnant rats from G12 to 21, and observed histopathological changes in fetal testes such as degeneration of seminiferous tubules, focal interstitial cell hyperplasia and adenoma at 500 mg/kg, but not at 100 mg/kg. Parks *et al.* (2000) treated maternal rats with 750 mg/kg of DEHP from G14 to postnatal day 3 and observed the appearance of multinucleated genocytes and hyperplasia of interstitial cells in the testis of G20 fetuses and in offspring at Day 3 of lactation. Thus, the present study has confirmed the characteristics of phthalate toxicity on testicular development in rats, which seems to occur in spite of differences in esterifying alcohol and administration protocol. The no-observed effect-level of DEHP on the testicular development of rats by *in utero* exposure during the period of organogenesis was 125 mg/kg. Target cells of the testicular toxicity of phthalates are the germ cells in the fetal rat, while they are the Sertoli cells in the adult rat when the blood-tes-

Photo 7. An electron micrograph of genital ridge of a rat fetus on gestation day 16 treated with 1000 mg/kg of DEHP, showing degenerated germ cells. ×2830.

tis barrier is established (Creasy *et al.*, 1983, Saitoh *et al.*, 1997, de Kretser and Kerr, 1994).

In the present study, EE was used as a reference compound, considering some interventions of estrogenic activity of DEHP for its toxicity on the testis. The result was negative for this consideration, although some relation may have existed to the increase in embryonic mortality. Estrogenic activity of various phthalate esters was investigated by Zacharewski *et al.* (1998). They observed weak estrogen receptor affinity *in vitro* for some phthalate esters other than DEHP, but no estrogenic activity *in vivo* for any of the phthalate esters by rat uterotropic assay. On the other hand, antiandrogenic activity has been suggested as one of the mechanisms of testicular toxicity of phthalate esters (Mylchreest *et al.*, 1998). Mylchreest *et al.* (1999) observed disturbances in male reproductive development with 500 mg/kg of DBP comparable to 100 mg/

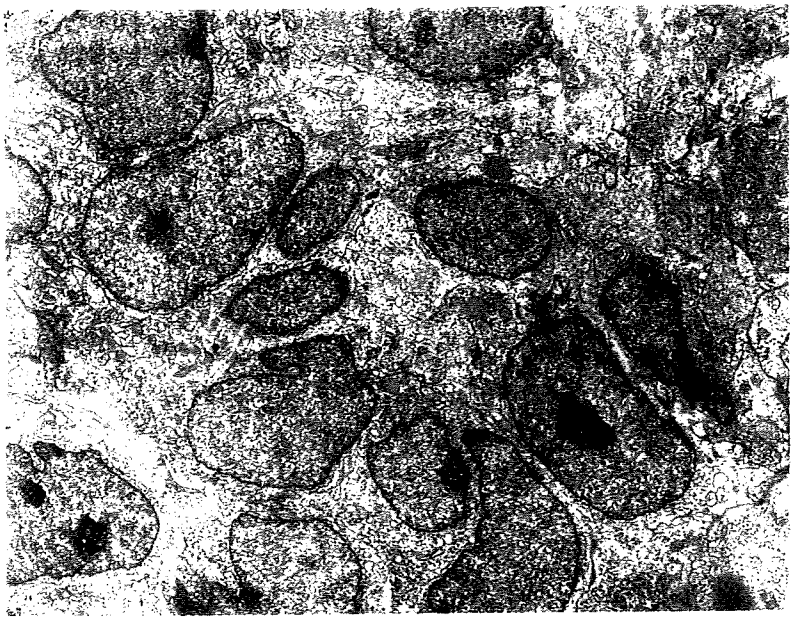

Photo 8-a. An electron micrograph of testis of a rat fetus on gestation day 18 treated with 1000 mg/kg of DEHP, showing decreased number of lipid droplets in small-sized interstitial cells. ×3140.

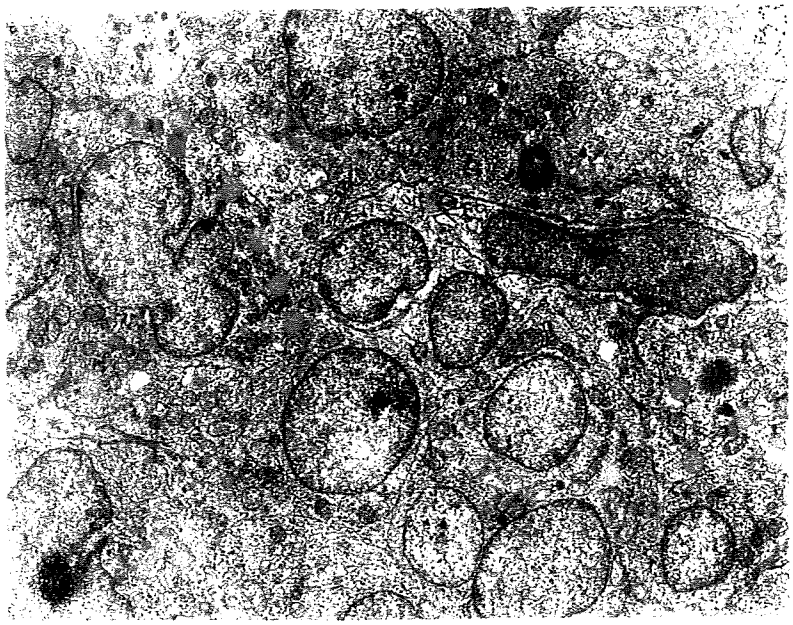

Photo 8-b. An electron micrograph of testis of a rat fetus on gestation day 20 treated with 1000 mg/kg of DEHP, showing decreased number of lipid droplets in small-sized interstitial cells. ×3140.

kg of flutamide, a known anti-androgen, but they could not confirm any interaction of phthalate with androgen receptor *in vitro*. They explained that DBP exerted its anti-androgenic activity by indirectly interfering with androgen signaling pathways (Mylchreest and Foster, 2000). Parks *et al.* (2000) observed inhibition of testosterone production of fetal testis (G17-20) with DEHP (750 mg/kg) in the experiment cited above. In the present study, increase of androgen receptor-positive interstitial cells was observed in G20 fetal testis in the groups treated with DEHP at 250 mg/kg and above. It is conceivable that interstitial cells and androgen receptors are increased by compensatory responses to reduced testosterone levels. Thus, anti-androgenic activity of DEHP is suggested from the observation of the present study, although malformations of male genital organs typical of anti-androgens such as flutamide (Mylchreest *et al.*, 1998, 1999) were not observed with DEHP up to 1000 mg/kg in the present study.

The present study has demonstrated that testicular damage in fetal rats produced by DEHP at 500 mg/kg (but not at 1000 mg/kg) had been repaired by 7 weeks of age. This was confirmed in the second experiment at 5 and 10 weeks of age. Expression of androgen receptors in testicular cells was normal in these stages of rats. Moreover, examination of sperm in off-

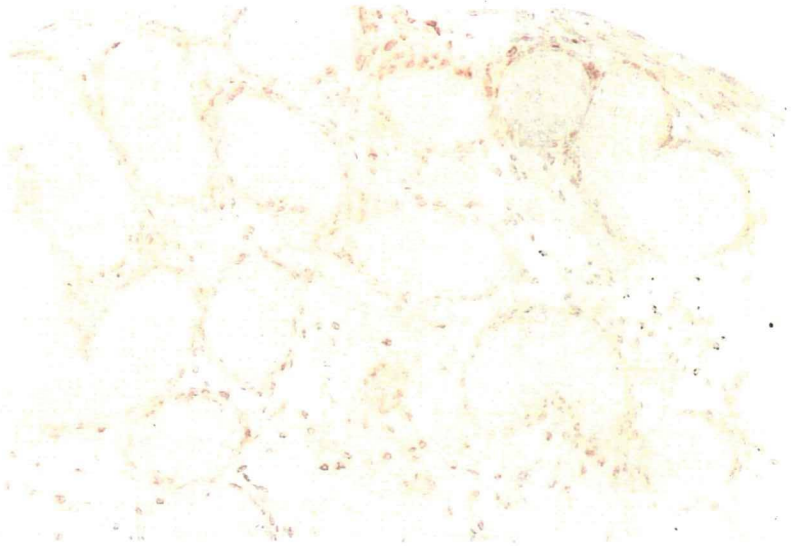

Photo 9-a. Immunohistochemical staining of androgen receptors in testis on rat fetus on G20 from the control group. Positive signals are observed on peritubular myoid cells and interstitial cells. ×175.

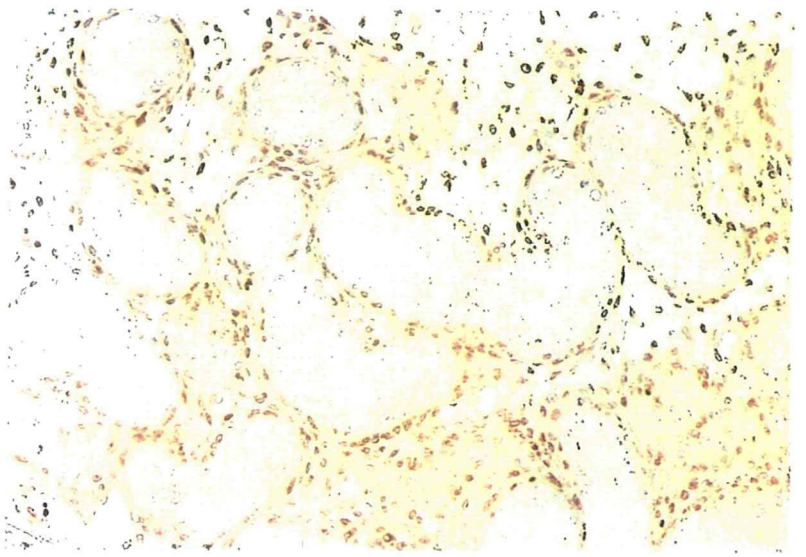

Photo 9-b. Immunohistochemical staining of androgen receptors in testis of rat fetus on G20 from the group treated with 500 mg/kg of DEHP. Interstitial cells with positive androgen-receptor signals are increased. ×175.

spring of DEHP-treated rats at 10 weeks of age showed no abnormal features of sperm function and morphology.

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Table 8. Examination of epididymal spermatozoa at 10 weeks after birth in the offspring exposed to di-(2-ethylhexyl) phthalate (DEHP) during gestational days 7-18 (Experiment 2).

	DEHP (mg/kg)			
	0 [a]	125	250	500
Animals examined	4	4	4	4
Sperm counts (per cauda epididymis) [b]	176.7 ± 55.7	142.9 ± 51.2	149.9 ± 48.9	175.0 ± 49.8
Sperm counts/cauda epididymis weight (g) [b]	1015.2 ± 241.0	878.0 ± 305.6	872.9 ± 198.7	992.1 ± 335.2
Sperm motility				
Rate of motile sperm (%) [b]	98.1 ± 1.2	96.6 ± 1.0	98.4 ± 1.1	97.2 ± 1.8
Rate of progressive sperm (%) [b]	84.4 ± 5.2	85.5 ± 1.8	88.7 ± 2.0	88.4 ± 3.2
Sperm morphology				
Sperms examined	800	800	800	800
Sperms with abnormalities	34	49	45	44
Abnormality rate (%) [b]	4.3 ± 1.7	6.1 ± 1.7	5.6 ± 5.5	5.5 ± 1.8
Types and incidence (%) of abnormal sperms				
Pin head	0	0	0.3	0.3
Amorphous head	0	0	0.1	0
Short head	0.1	0	0	0.1
Banana head	0	0.1	0	0
Reduced hock	0.1	0.5	0.4	0.3
No hock	0	0.1	0.1	0.1
Excessive hock	0	0	0.1	0
Bent flagellum	0.1	0.1	0	0
Broken flagellum	0.1	0.5	0.1	0.4
Bent neck	0.4	0.6	0.1	0.6
Isolated head	3.3	4.1	4.4	3.8
Two heads, one tail	0.1	0	0	0

[a] Vehicle control (corn oil, 5 mL/kg). [b] Values represent mean ± S.D.

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SUBCELLULAR DISTRIBUTION OF DI-(2-ETHYLHEXYL)PHTHALATE IN RAT TESTIS

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ABSTRACT — Subcellular distribution of di-(2-ethylhexyl)phthalate (DEHP) in the testis was studied by single oral administration of [3,4,5,6-^{3}H]-phthalic acid di-(2-ethylhexyl) ester (DEHP-^{3}H) or phthalic acid di-(2-ethyl[1-^{3}H]hexyl) ester (^{3}H-DEHP) to 8-week-old male rats. Autoradiographs and electron microscopic autoradiographs were prepared from the testis, liver and kidney at 6 and 24 hr after administration and distribution of radioactive materials in the tissues were observed. In the autoradiographic specimen at 6 hr after administration of DEHP ^{3}H-labeled at phthalic acid moiety (DEHP-^{3}H), many grains were observed in the testis, mainly at the basal area of seminiferous tubules at the stages IX to I of the spermatogenic cycle. Electron microscopic autoradiographs taken at the same time revealed that localization of grains were in the smooth-surfaced endoplasmic reticulum and mitochondria of Sertoli cells. A few grains were also present at the Golgi apparatus and lysosome of Sertoli cells, and at the interfaces between the Sertoli cells or between Sertoli cells and spermatocytes, and in the cytoplasm of spermatocytes. Autoradiographs of the liver revealed grains in the centrilobular hepatocytes, localized at mitochondria, rough-surfaced endoplasmic reticulum and peroxisomes. In the kidney, the radioactivity was localized at the brush border of the tubular cells in the pars recta of proximal tubules. In the 24-hr specimen, the grain density in the seminiferous tubules obviously decreased. On the other hand, by autoradiography with DEHP ^{3}H-labeled at the alcohol (^{3}H-DEHP), only a few grains were observed in autoradiographs of the testes at 6 hr after administration. No grains were noted in autoradiographs of the liver and kidney with ^{3}H-DEHP. The results showed that the phthalic acid ester was splitted rapidly in the body and only the phthalic acid moiety distributed into the cells.

KEY WORDS: Phthalic acid ester, Autoradiography, Electron microscopy, Testicular toxicity, Sertoli cells, Rats

INTRODUCTION

It has been shown that high doses of phthalic acid esters exert testicular toxicity in animals (Calley *et al.*, 1966; Gangolli, 1982). The toxic effect on the testis was similarly observed with a variety of phthalate esters such as di-(2-ethylhexyl) phthalate (DEHP) (Gray *et al.*, 1977), dibutylphthalate, (Cater *et al.*, 1977) and di-n-pentyl phthalate (Creasy *et al.*, 1987). Among a variety of phthalate esters, DEHP has been investigated most frequently as a representative substance of phthalic acid esters. The mechanism of the testicular toxicity of phthatates is not yet wholly clear, although the effect on Sertoli cells and damaging blood-testis barrier has been considered (Gray and Butterworth, 1980). We have conducted a series of experiments on testicular toxicity of DEHP in rats, and have clarified that ultrastructural changes were induced in seminiferous tubules at the stages from IX to I of the spermatogenic cycle in 8-week-old Sprague-Dawley rats, 3 to 18 hr after single dose administration of 2800 mg/kg of DEHP (Saitoh *et al.*, 1997). Noteworthy

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changes were degeneration of spermatocytes, dilatation of rough-surfaced endoplasmic reticulum, especially those in the vicinity of the tight junction of ectoplasmic specialization of Sertoli cells, and disintegration of the intercellular junction between Sertoli cells. We have also observed that clear structural changes of testes were induced with a single oral dose of 1400 mg/kg, and that the non-toxic dose level of DEHP on testes was 700 mg/kg in mature rats. Furthermore, we have employed a lanthanum trace method to examine the effects of DEHP on Sertoli cell function, especially on the condition of the blood-testis barrier in rats (Saitoh *et al.*, 1997). In this study, lanthanum particles were observed 6 hr after administration at the tight junction between Sertoli cells. The number of particles observed reached its peak from 18 to 24 hr after administration, and then it showed a slight decrease. Therefore, it was concluded that the function of Sertoli cells to maintain the blood-testis barrier was affected by DEHP as early as 6 hr after oral administration, but was recovering by 24 hr. Gray and Gangoli (1986) have reported that oral DEHP is hydrolyzed in the intestine to mono(2-ethylhexyl)phthalate (MEHP), which is then absorbed and exerts testicular toxicity. However, they found very limited transfer of radioactivity into the testicular tissue in spite of its testicular toxicity after administration of ^{14}C-MEHP labeled at 7th position of the side chain (Gray and Gangoli, 1986; Albro *et al.*, 1973). In the present study, two kinds of ^{3}H-labeled DEHP, labeled either at the phthalic acid or at the alcohol moiety, were used to elucidate its distribution in the testis by autoradiography, including electron microscopic observation. In addition, subcellular distribution of these labeled compounds in the liver and kidney were studied.

MATERIALS AND METHODS

Materials

Two kinds of ^{3}H-labeled DEHP were purchased from Amersham Biosciences and used for this study. One was labeled at the phthalic acid ring ([3,4,5,6-^{3}H]-phthalic acid di(2-ethylhexyl) ester; specific activity: 1.85 TBq/mmol, hereafter referred to as DEHP-^{3}H) and the other was labeled at the alcohol moiety (phthalic acid di(2-ethyl[1-^{3}H]hexyl)ester; specific activity: 1.22 TBq/mmol, hereafter referred to as ^{3}H-DEHP). Toluene was removed from the commercial solutions of each labeled compound with nitrogen stream, and then each compound was suspended in corn oil (Nakalai Tesque). Standard DEHP was purchased from Wako Pure Chemical Industries.

Animals

Male rats of the Sprague-Dawley strain (Crj: CD, SPF) were purchased at 5 weeks of age from Atsugi Breeding Center, Charles River Japan, Inc., and were kept for three weeks to acclimatize to laboratory condition. The animals were reared in an individual cage sized 235×395×170 mm and kept in a glass-front animal isolator with conditioned air supply of temperature at 24~26°C and relative humidity within 50~65%. The animals were fed with pellet food (CE-2, CLEA Japan Inc.) *ad libitum* and were supplied with tap water. They were allocated to two groups of four animals each in a random fashion on the day before administration.

Dosage and administration

As we have observed in a preceding study (Saitoh *et al.*, 1997), slight but obvious ultrastructural changes of rat testis with a single oral dose of 1000 mg/kg of DEHP, the dose of DEHP in the present study was determined to be 1000 mg/kg. The dose was adjusted by adding the standard and a radioactive DEHP and the solution volume adjusted to 5 mL/kg, which contained radioactivity of 92.5 MBq. The test solution was prepared as described above and oral administration was performed by gavage.

Autoradiography

Specimens for autoradiography were collected from each of the two animals in both groups at 6 and 24 hr after administration of radio-labeled DEHP. The chest of the animal was opened by median thoracotomy under pentobarbital anesthesia. A cannula was inserted from the apical part of the left ventricle along the interventricular septum through the aortic outflow tract into the aorta, and was fixed there by ligation around the aorta. Phosphate-buffered saline was infused through the cannula to the body, keeping the perfusion pressure between 700-800 mmH_2O by adjusting the rotating speed of the infusion pump. Immediately after beginning the infusion, the right atrium was sectioned. When the outflow from the right atrium became very transparent (about one minute after), the flushing solution was switched to a combined fixative solution of 0.1 M phosphate buffered 2% paraformaldehyde and 1.25% glutaraldehyde (pH 7.4). After the fixative solution was perfused for 15 to 20 min, the testes, liver and kidneys were collected from the body. The collected organs were cut and postfixed in 0.1 M phosphate buffered 2% osmium tetroxide.

After being dehydrated in serial ethanol solutions of graded concentration, the tissues were embedded in Quetol-651 resin block. Semi-thin sections, about 1 μm thick, were prepared from each block. The sections were dipped into radiographic emulsion Type NTB-2 (Eastman Kodak) and exposure to tissue radioactivity maintained for four or seven days in a dark cabinet at 4°C. After development using Kodak Dektol (Eastman Kodak) and fixation with Fujifix (Fuji Photo Film), each slide was stained with toluidine blue. These autoradiographic slides were examined under a light microscope.

For the testis, grains were counted for every seminiferous tubule in three slides for each sampling time, and progress of grain count with time was examined. For evaluation of accumulation of radioactivity, the mean background grain count and twice standard deviation was reduced from the mean grain count per seminiferous tubule.

Parts of some testes that showed rich grain distribution in seminiferous tubules on the autoradiography were dissected out for ultrastructural observation. Ultrathin sections were made of these parts, placed on a slide glass pretreated with collodion, stained with uranyl acetate and lead citrate, and coated with evaporated carbon. Then the sections were dipped into electron microscope autoradiographic emulsion Type L4 (Ilford), and left exposed to radioactivity for five or eight weeks in a dark cabinet at 4°C. After development using fenidone, a thin layer of collodion coat was stripped off, and the ultrathin section was dried under a grid. Then the slide was observed with an electron microscope (H-7100, Hitachi).

RESULTS

Autoradiography by single oral dose of DEHP-^{3}H

1. Autoradiography under a light microscope

1) Testes

In autoradiography of testes prepared at 6 hr after administration of DEHP-^{3}H, a considerable number of grains was observed in seminiferous tubules, mostly at the basal area of seminiferous tubules at the stages from IX to I of the spermatogenic cycle (Photo 1). The mean grain count per seminiferous tubule was 23. The grain count of this area decreased in the 24-hr autoradiographs (Photo 2). The mean grain count per seminiferous tubule was 12. Mean grain counts per seminiferous tubule corresponding to the spermatogenic stage are depicted in Fig. 1, which shows preferential distribution of the grains to stages IX to I of spermatogenesis at 6 hr after administration, and also shows the change of distribution by time.

2) Liver

In 6-hr autoradiographs of the liver, the distribution of radioactivity was at the background level. However, at 24 hr after administration, the grain count increased in the hepatocytes of the centrilobular area (Photo 3). The number of grains of this area of this period was higher than that in the testis at the peak.

3) Kidneys

In autoradiographs of the kidney at 6 hr after administration, many grains were noted at the brush border of epithelial cells of the straight proximal renal tubules and also at the luminal side of cytoplasm of the cells (Photo 4). However, in 24-hr autoradiographs, the grain count decreased to the background level.

2. Electron microscopic autoradiography

1) Testes

Observation by electron microscope was conducted specifically on the seminiferous tubules at the stages from IX to I of the spermatogenic cycle. At 6 hr after administration, grains were observed at the smooth-surfaced endoplasmic reticulum and mitochondria of Sertoli cells (Photo 5-A). Grains were also noted at the junctions between neighboring Sertoli cells or between a Sertoli cell and spermatocytes (Photo 5-B). Moreover, small amounts of grains were observed at the Golgi apparatus and lysosome of Sertoli cells, and also in the cytoplasm of spermatocytes. In 24-hr autoradiographs, similar subcellular distribution of grains to 6-hr autoradiographs were observed, showing some decrease of grains in mitochondria and increases in rough-surfaced endoplasmic reticulum of Sertoli cells and in spermatocytes and spermatid. Actual counts of grains are tabulated in Table 1 to show the subcellular distribution of the grain.

2) Liver

At 6 hr after administration, a few grains were observed at rough-surfaced endoplasmic reticulum, peroxisome, and mitochondria of the hepatocytes. At 24 hr after administration, grains were observed most frequently at mitochondria, and to a lesser extent at rough-surfaced endoplasmic reticulum and peroxisome (Photo 6).

3) Kidneys

Grains were observed at the brush border of epi-

thelial cells of the straight portion of proximal tubules, and at mitochondria, smooth-surfaced endoplasmic reticulum, peroxisome, and Golgi apparatus in 6-hr autoradiographs (Photo 7). However, in the 24-hr specimens, only a few grains were observed at the brush border, mitochondria, and peroxisome of the epithelial cells.

Autoradiography by single oral dose of ^{3}H-DEHP

1. Autoradiography under light microscopy

In autoradiographs of testes at 6 hr after oral administration of ^{3}H-DEHP, the grain count of the testes was only a little higher than the background level. The mean grain count per seminiferous tubule was 6, which is obviously fewer than the count of the corresponding autoradiographs with DEHP-^{3}H. No specific distribution of grains to the stage of the spermatogenic

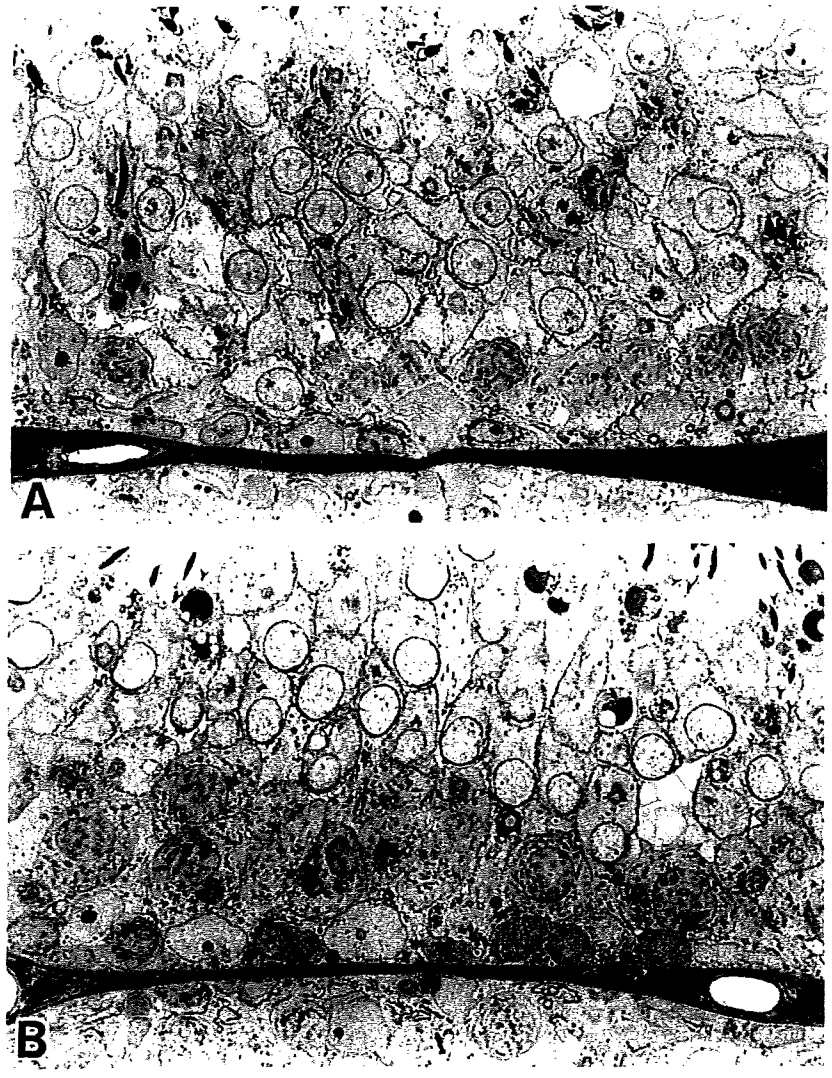

Photo 1. Light microscopic autoradiographs from the testis of a rat sacrificed 6 hr after administration of [3,4,5,6-^{3}H]-phthalic acid di(2-ethylhexyl)ester. Accumulation of silver grain is noted at the basal area of seminiferous tubules.
A: Stage I seminiferous tubule, × 800.
B: Stage IX seminiferous tubule, × 800.

Distribution of DEHP in rat testis.

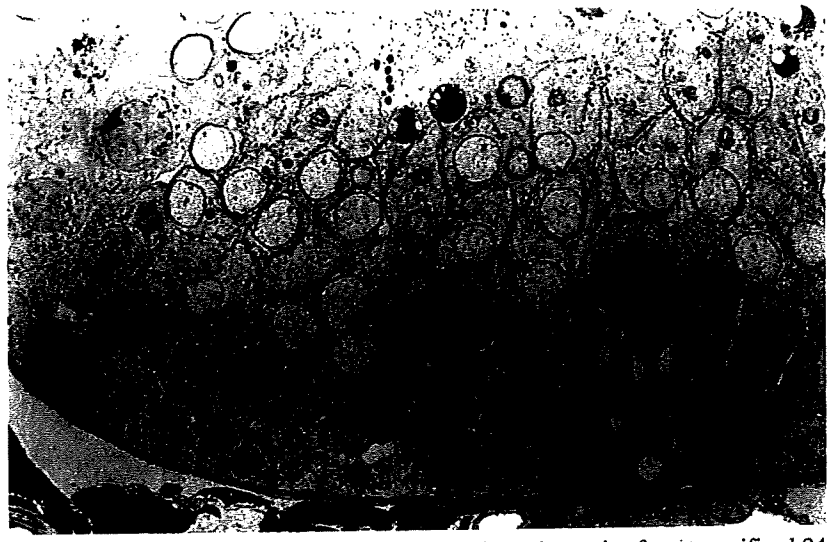

Photo 2. A light microscopic autoradiograph from the testis of a rat sacrificed 24 hr after administration of [3,4,5,6-^{3}H]-phthalic acid di(2-ethylhexyl)ester. A few silver grains are noted at the basal area of a seminiferous tubule at stage IX, × 800.

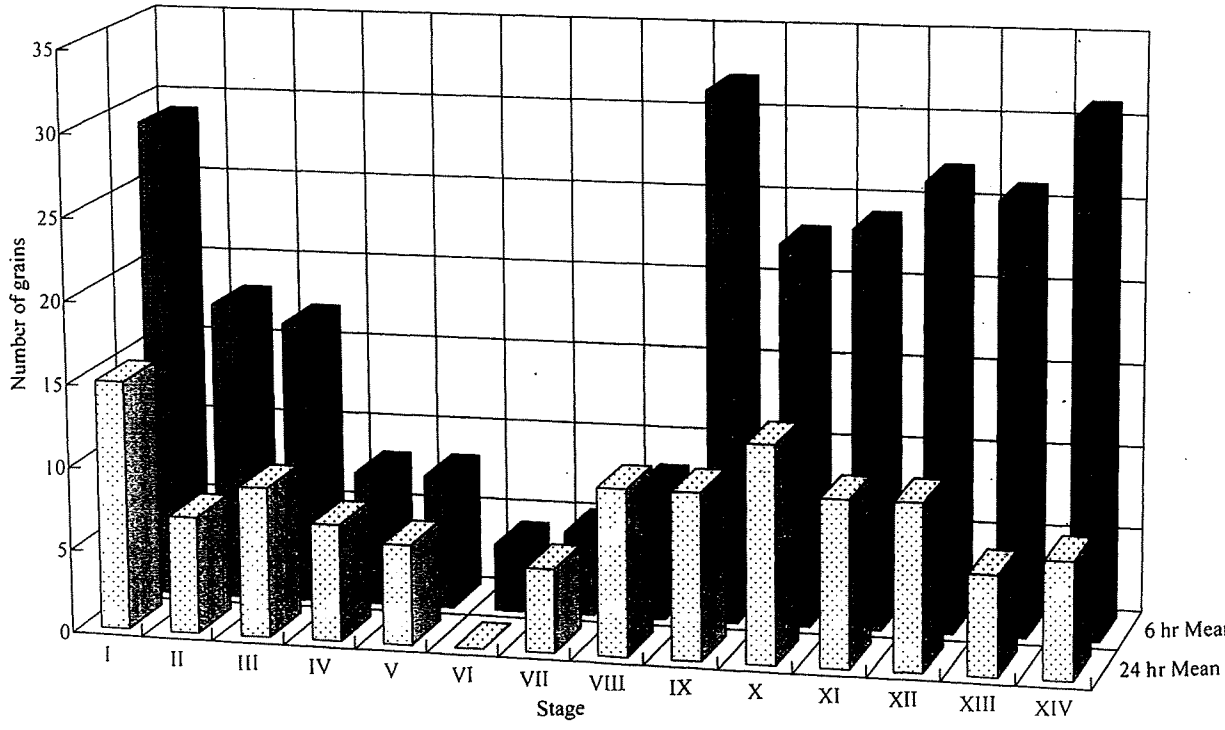

Fig. 1. Distribution of radioactivity to seminiferous tubules of the testis of rats 6 and 24 hr after administration of [3,4,5,6,^{3}H]-phthalic acid di(2-ethylhexyl)ester. Mean counts of radiosensitized grains per seminiferous tubule on the autoradiographs are shown by the stage of spermatogenesis.

cycle was observed. The grain count in 24-hr autoradiographs was at the background level. Fig. 2 shows the distribution of grain count in the testis on 6-hr and 24-hr autoradiographs.

2. Electron microscopic autoradiography

In either 6-hr or 24-hr autoradiographs, no grains were observed in any specimens of testes, liver, or kidneys by electron microscopy.

DISCUSSION

In the present study, a single dose of DEHP (1000 mg/kg), labeled with ^{3}H at the phthalic acid or at the

Photo 3. A light microscopic autoradiograph from the liver of a rat sacrificed 24 hr after administration of [3,4,5,6-^{3}H]-phthalic acid di(2-ethylhexyl)ester. A few grains are noted on the hepatocytes, × 800.

Photo 4. A light microscopic autoradiograph from the kidney of a rat sacrificed 6 hr after administration of [3,4,5,6-^{3}H]-phthalic acid di(2-ethylhexyl)ester. Many silver grains are noted at the luminal border of epithelial cells of the straight part of proximal tubule, × 800.

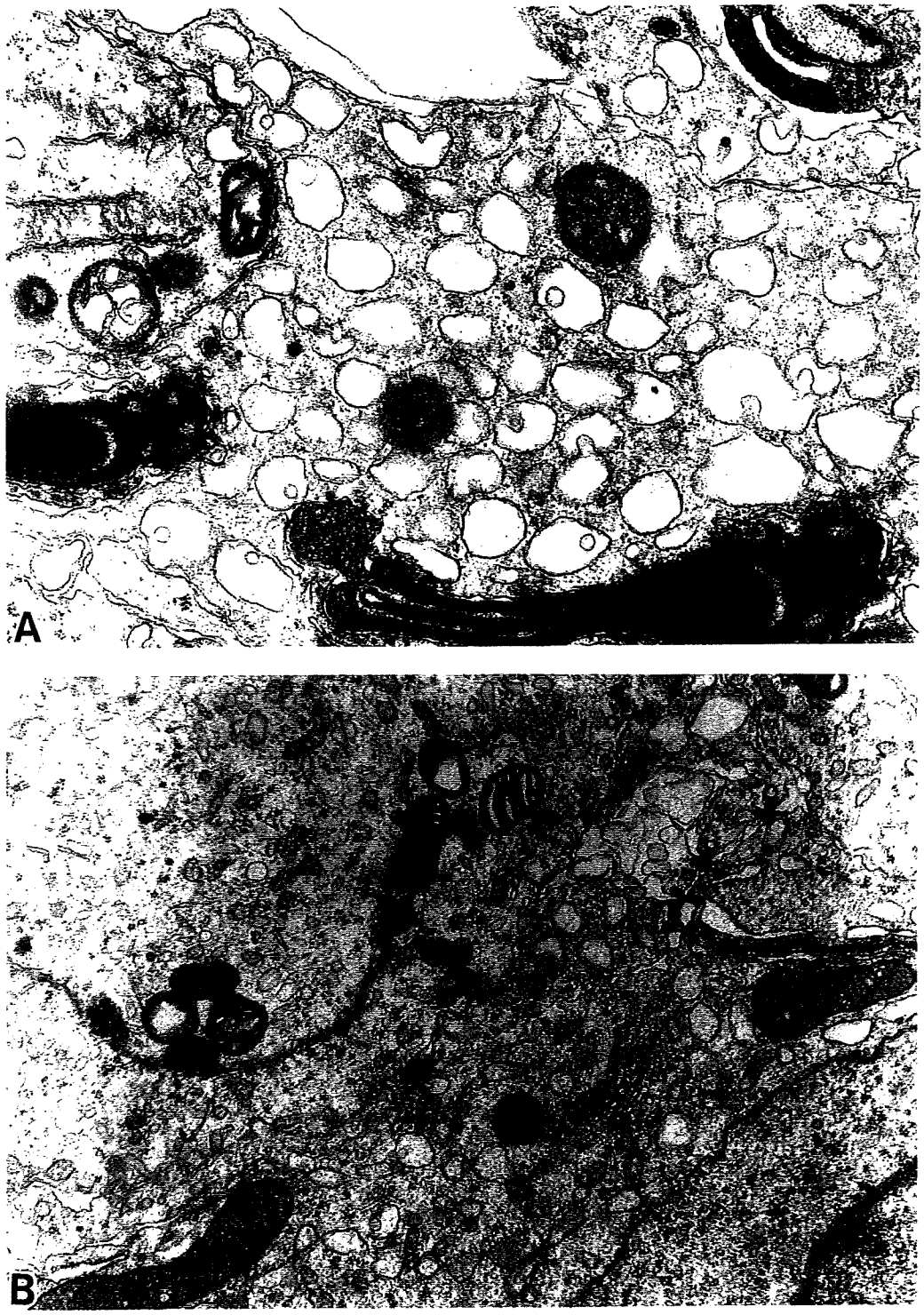

Photo 5. Electron microscopic autoradiographs from seminiferous tubules of testis sacrificed 6 hr after administration of [3,4,5,6-^{3}H]-phthalic acid di(2-ethylhexyl) ester.
A: A few silver grains are noted on the mitochondria and smooth-surfaced endoplasmic reticulum of Sertoli cell, × 22800.
B: A few silver grains are noted on the extracellular space. Seminiferous tubule at stage I of spermatogenesis, × 17100.