

# Necrotizing Effect of Ethanedimethanesulfonate on Spontaneously Occurring Leydig Cell Tumors in Old F344 Rats

Sotokichi Morii,<sup>1</sup> Yuji Naka, Toshihiko Inui, and Hiroshi Shintani

Departments of Pathology [S. M., T. I.] and Urology [Y. N., H. S.], Kansai Medical University, Moriguchi, Osaka 570, Japan

## ABSTRACT

Thirty 18-month-old male F344/DuCrj rats were divided into the following groups: 10 untreated controls; eight vehicle-injected controls; and 12 ethanedimethanesulfonate (EDS)-injected rats. Untreated controls were killed immediately to check for testicular tumor incidence. In rats of the test group, a 75-mg/kg dose of EDS dissolved in dimethyl sulfoxide:water (1:3) was injected i.p. At intervals of 1, 2, 3, and 10 days after injection, two vehicle-injected control rats and three EDS-injected rats were sacrificed, and the testes were fixed by vascular perfusion. The midsagittal sections of all the fixed testes were examined to determine the incidence of macroscopic Leydig cell tumors, and some tumor tissues of the injection-treated groups were also investigated ultrastructurally. In 28 of 30 animals, a total of 78 Leydig cell tumors could be distinguished. Extensive and severe necrotic alterations accompanying fresh, multiple hemorrhages in early stages and reparative changes in later stages could be observed in a total of 78% of the 32 tumors examined from the EDS-injected group. The tumor cells exhibited ultrastructurally degenerative changes such as chromatin condensation and cytoplasmic vacuolation from 1 day after EDS injection. Therefore, EDS may be a necrotic agent for rat Leydig cell tumor.

## INTRODUCTION

EDS<sup>2</sup> was one of a homologous series of alkylating agents investigated for their potential use in the chemotherapy of neoplasm (1). After a single injection of EDS (75 mg/kg), secretion of androgen by the testis in rats was greatly diminished within 3 days and remained low for 3 weeks, with full recovery by 7 weeks (2, 3). Several reports have indicated that a transient decrease in the amounts of testosterone observed after treatment with a dose of EDS was due to the destruction of Leydig cells (4). Recently, ultrastructural changes in stromal tissues of the testis have been described in the rats given injections of EDS (5, 6). Apparently, this compound is a specific toxin for rat Leydig cells and hence will destroy Leydig cell tumors in rats. Despite these intriguing observations, the *in vivo* effects of EDS on tumors originating from Leydig cells have not yet been documented. In this communication, the necrotizing effect of EDS on spontaneously occurring Leydig cell tumors in old Fischer rats is described.

## MATERIALS AND METHODS

Male F344/DuCrj rats were purchased from the Japan Division of Charles-River Breeding Laboratories and maintained in our animal room. At the age of 18 months, 30 rats weighing 330-400 g were divided randomly into the following experimental groups: 10 untreated controls; eight vehicle-injected controls; and 12 EDS-injected rats. When the untreated controls reached the age of 550 days, they were killed by decapitation and their testes were fixed in 10% buffered formalin prior to inspection for testicular tumors. Although no abnor-

mality in the testis could be detected by palpation before the decapitations in all animals, one to two nodular tumors were found in cut surfaces of the fixed testes in nine rats (tumor incidence, 90%). These were diagnosed as Leydig cell tumors. Therefore, at the age of 556 days, two rats from the vehicle-injected control group and three rats from the EDS-injected group were sacrificed 1 day after an i.p. injection. On the 2nd, 3rd, and 10th days, respectively, at the age range of 557-565 days, a similar number of animals from each injection-treated group were killed. EDS is not commercially available and was synthesized by Ishizu Chemical Co., as described by Jackson and Jackson (7). EDS was dissolved in DMSO:distilled water (1:3) to give a concentration of 25 mg/ml. The solution was injected i.p. into 12 rats as a single dose of 75 mg/kg. Concurrently, the controls were given approximately 1 ml of the diluted DMSO. The testes of all the injection-treated rats were fixed by vascular perfusion per the method of Forssmann *et al.* (8), except that one extra ligature was placed around the left renal artery at the renal hilum, in addition to the three ligatures suggested by them. After an initial washout solution containing a vasodilator and an anticoagulant was perfused for 1 min, a phosphate-buffered, 3.3% glutaraldehyde solution was perfused for 10 min. The perfused testes were cut midsagittally and fixed again in the same fixative for 3 h. Approximately 1-mm<sup>3</sup> tissue blocks from one-half of the tumors found at each time point were postfixed in 2% osmium buffer for 2 h, dehydrated in graded ethanols, and then embedded in epoxy resin. The macroscopic appearance of the sagittally cut surfaces of all the fixed testes was examined for nodular tumors. All such tumors were examined histologically. Ultrathin sections were stained by the usual method with metals and viewed under a Hitachi-500 electron microscope.

## RESULTS

The combined average weight of both testes in 18-month-old rats,  $3.8 \pm 0.43$  g, (SD) represents a slight increase over that of 12-month-old rats ( $3.3 \pm 0.41$  g). Any testis bearing large tumors was enlarged at the expense of the testis, which was atrophied. However, weights of those fixed in this experiment were not significantly different from each other. Miliar and/or pea-sized, pinkish, nodular tumors were detected macroscopically in midsagittal sections of the fixed testes. Double or confluent tumors were recognized infrequently in the testes examined, while bilateral tumors were quite often noted. Finally, it was found that 28 rats had a total of 78 Leydig cell tumors, thus giving a tumor incidence of 93.3%. No significant correlation was apparent between tumor occurrence and the weight of combined accessory sex organs ( $747.5 \pm 40.85$  mg). The paired testis weight of EDS-injected rats was not significantly different from that of vehicle-injected rats at any time during the experiment. Within 3 days of EDS-injection, however, gray necrotic foci contaminated with dark red hemorrhages were observed in 19 of 24 tumors found within 18 testes of the EDS-injected rats. Brownish gray foci could be detected in six of eight tumors found within six testes of the EDS-injected rats on the 10th day. In the vehicle-injected group no necrotic foci could be seen in 20 nodular tumors found within 14 testes in the group as well as in a rat having no Leydig cell tumors.

Histological examinations showed that the nodular tumors were composed of large cells with abundant eosinophilic, vacuolated cytoplasm and nuclei typical of those of actively secret-

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<sup>1</sup> To whom requests for reprints should be addressed, at the Department of Pathology, Kansai Medical University, Fumizonochi 1, Moriguchi, Osaka 570, Japan.

<sup>2</sup> The abbreviations used are: EDS, ethanedimethanesulfonate; DMSO, dimethyl sulfoxide; LH, luteinizing hormone.

ing Leydig cells (Fig. 1) and/or smaller cells with scanty, pale cytoplasm and a dense, wheel-like nuclear pattern typical of unstimulated interstitial cells of intertubular tissues in the testis (Fig. 2). Such differing histological patterns could often be detected within a testicle, and even within the same nodule, where an intermediate cell pattern could sometimes be seen. Microcysts, minute necroses, and small hemorrhages were occasionally observed in the tumor tissues of the control groups. In general, larger tumors were accompanied by severe atrophy of the surrounding seminiferous tubules, while smaller ones were found within the active testicular tissues. Nontumorous Leydig cells of the control groups were slightly atrophic generally, but some were focally hyperplastic. On the other hand, in the EDS-injected group, extremely large and severe necrotic alterations were demonstrated in 19 of the 24 Leydig cell tumors examined in 18 testes of nine rats at the 1st, 2nd, and 3rd days of EDS administration. Such necrosis was accompanied by fresh, multiple hemorrhages and leukocytic infiltrations (Figs. 3 and 4), with similar degenerative and necrotic changes being seen also in most of the nontumorous Leydig cells. Subacute stromal reactions (hemosiderin-containing macrophages, proliferating fibroblasts, fibrosis, etc.) were observed in the peripheral areas of completely necrotic foci in six of eight Leydig cell tumors on the 10th day of EDS administration. At that time no Leydig cells could be detected in the intertubular tissues of six examined testes.

Neoplastic cells with two distinct types of ultrastructural characteristics were evident in the Leydig cell tumors of vehicle-

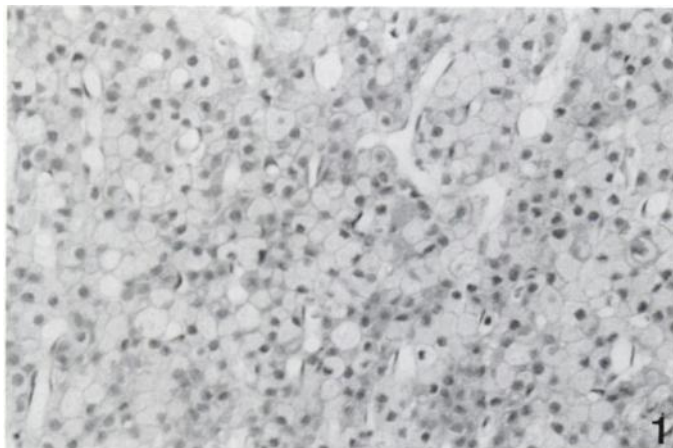


Fig. 1. Leydig cell tumor of a vehicle-injected control rat. Large cell pattern. H & E,  $\times 200$ .

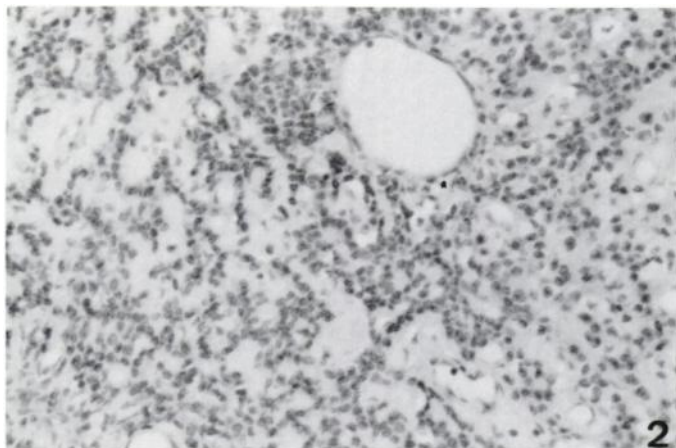


Fig. 2. Leydig cell tumor of a vehicle-injected control rat. Small compact cell pattern. H & E,  $\times 200$ .

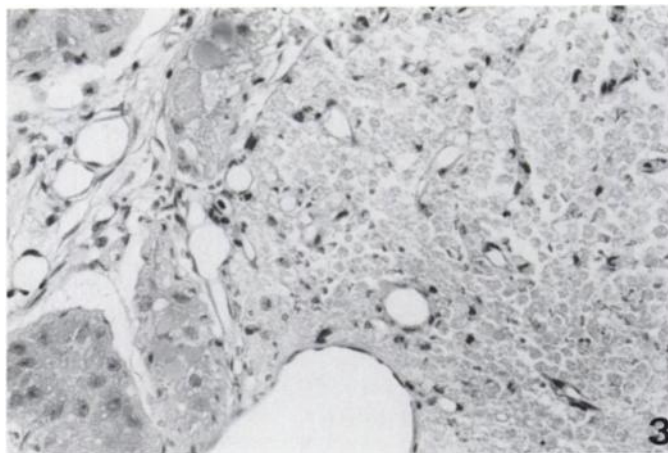


Fig. 3. Leydig cell tumor of a rat given an injection of EDS 2 days before. Severe necrosis can be seen in the tumor tissue, and some Leydig cells in the intertubular tissues are degenerative although the seminiferous tubules themselves are not injured. H & E,  $\times 200$ .

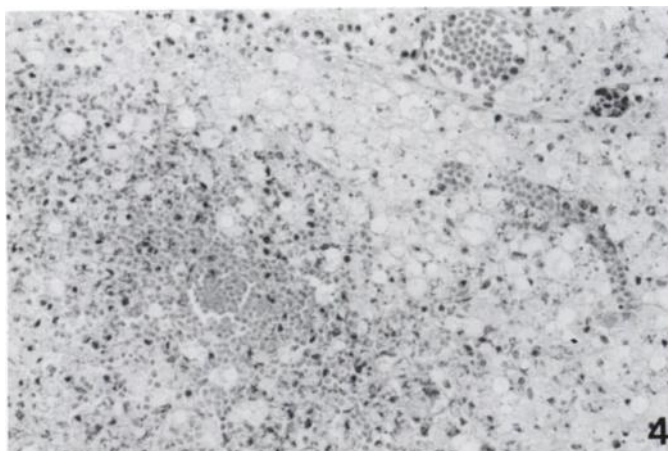


Fig. 4. Leydig cell tumor of a rat given an injection of EDS 3 days before. Large hemorrhages and extensive leukocytic infiltration can be seen in the necrotic focus. H & E,  $\times 200$ .

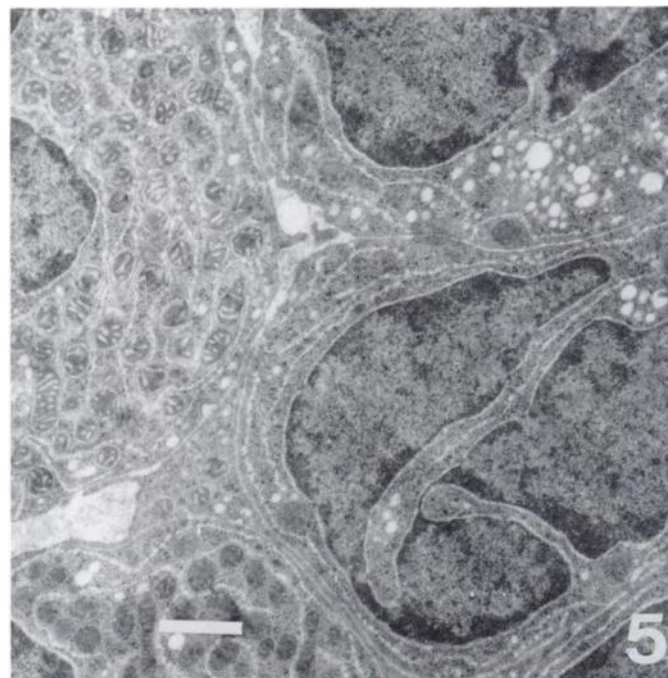
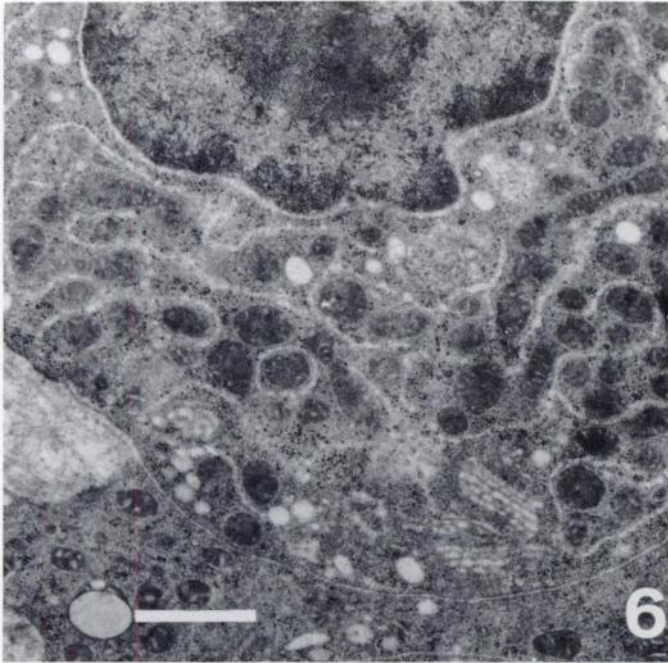
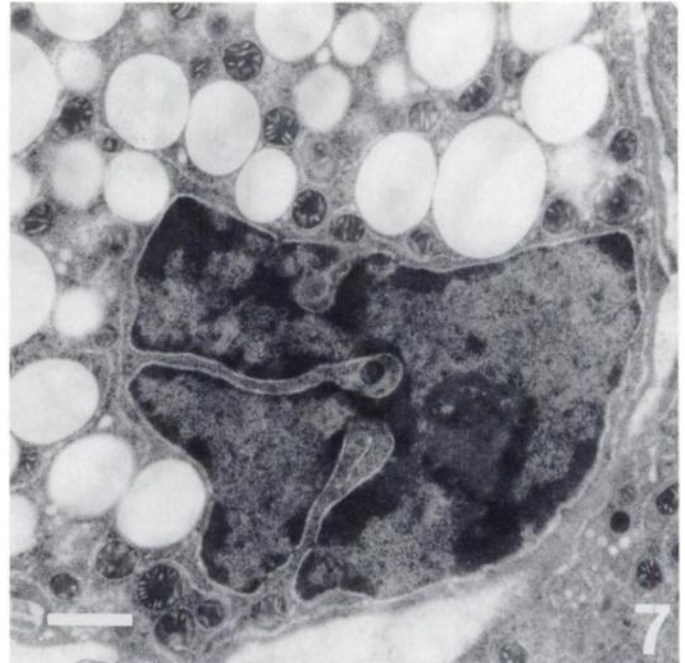


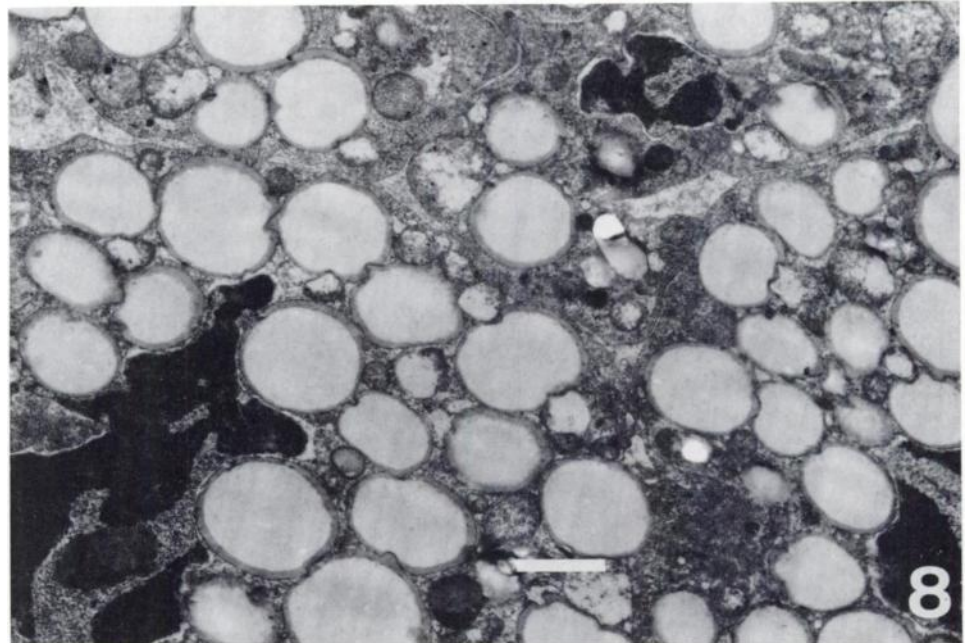
Fig. 5. Electron micrograph of Leydig cell tumor in a vehicle-injected control rat. Prominent nuclei, smooth ER, and tubular-structured mitochondria can be seen in the steroid secreting cells.  $\times 13,000$ .



**Fig. 6.** Electron micrograph of Leydig cell tumor in a vehicle-injected control rat. Free ribosomes, smooth ER, irregularly located Golgi bodies, and tubular-structured mitochondria can be easily seen in the cytoplasm.  $\times 21,000$ .



**Fig. 7.** Electron micrograph of Leydig cell tumor in a vehicle-injected control rat. An irregular and large nucleus can be seen in the cytoplasm where many lipid droplets, small mitochondria with vesicular cisternae, and smooth ER are prevalent.  $\times 13,500$ .



**Fig. 8.** Electron micrograph of Leydig cell tumor in a rat given an injection of EDS 1 day before. Masses of irregularly clumped chromatin are dispersed in the periphery of pyknotic nuclei. Marked vacuolar changes can be seen in the disorganized cytoplasm.  $\times 13,500$ .

injected controls. One cell type was characterized by small nuclei and scanty cytoplasm with many ribosomes, few smooth ER, and small mitochondria (Figs. 5 and 6). The other displayed the nuclei and cytoplasmic characteristics of actively steroid-secreting cells, where numerous profiles of lipid droplets, vesicular smooth ER, and mitochondria with vesicular cisternae could be seen (Fig. 7). On the first day of EDS administration, when the cytoplasm of most tumor cells appeared to be vacuolated, irregularly spherical masses of clumped chromatin were found dispersed around the periphery of the nuclei (Fig. 8). Many of the tumor cells seemed very dense and large granules appeared in the cytoplasm with infrequent ribosomes and mitochondria on the 2nd and 3rd days of EDS administration. At

that time, the nuclei were very pyknotic or lytic, and leukocytes and macrophages were prevalent. Around the necrotic foci in Leydig cell tumors of the rats given injections of EDS 10 days before, many tumor cells were unidentifiable and some displayed dense morphology characteristic of degeneration.

#### DISCUSSION

Leydig cell tumor is the most common testicular neoplasm in some strains of male rat (9). It occurs usually in old rats and the incidence increases with age. In the present experiment the incidence of macroscopic tumors in sagittal sections was 93% in the testis of 18-month-old Fischer rats. The growth rate of

this benign tumor has been reported to be mainly hormone responsive, especially LH dependent, but few of these tumors produce androgen (10). In the tissue of this benign neoplasm, microcysts, minute necroses, and small hemorrhages can sometimes be detected. However, a high incidence of extremely large necroses with multiple hemorrhages was discovered in the rats given injections of EDS during the present investigation. Degenerative changes in the ultrastructure of most Leydig cells of adult rats have been noted 12 h after a single 100-mg/kg dose of EDS (5). At 72 h after a single 75-mg/kg dose of EDS, no Leydig cells could be detected and no  $3\beta$ -hydroxysteroid dehydrogenase- or esterase-positive cells were present (5). Other compounds with structures similar to that of EDS, such as butanedimethylsulfonate and ethanemethylsulfonate, have had no effect on Leydig cells in mature rats (10). It has been concluded that EDS specifically destroys rat Leydig cells, and it is further proposed in this study that EDS is a necrotic agent for tumors which originate as Leydig cells. However, few Leydig cell tumors were refractory to the necrotizing action of EDS in our observations. While EDS is selective for Leydig cells of the mature rat testis, those of immature (22-day-old) male rats were resistant to EDS (11). Recently, acute response to EDS has been reported resulting in the degeneration of fetal rat Leydig cells and, via the phagocytic activity of interstitial macrophages, all fetal Leydig cells eventually disappeared from the testis (12). EDS had no detectable effect on steroid secretion in *in vitro* cultured cells from the Leydig cell tumors of rats (10). The cytotoxicity of EDS towards Leydig cells may be dependent on the extent of LH stimulation, and EDS may inhibit specifically LH-regulated functions of Leydig cells, possibly via alkylation of proteins. Further investigations should be carried out on

other LH-dependent tumors and on the Leydig cells of other species.

## REFERENCES

1. Jackson, H., and Craig, A. W. Effects of alkylating chemicals on reproductive cells. *Ann. NY Acad. Sci.*, **160**: 215-227, 1969.
2. Cooper, E. R. A., and Jackson, H. Comparative effects of methylene, ethylene and propylene dimethane sulphonates on the male rat reproductive system. *J. Reprod. Fertil.*, **23**: 103-108, 1970.
3. Morris, I. D., and McCluckie, J. A. Temporal changes in serum androgen after temporary impairment of Leydig cell function by ethane-1,2-dimethane sulphonate. *J. Steroid Biochem.*, **10**: 467-469, 1979.
4. Molenaar, R., de Rooij, D. G., Rommerts, F. G., Reuvers, P. J., and van der Molen, H. J. Specific destruction of Leydig cells in mature rats after *in vivo* administration of ethane dimethyl sulfonate. *Biol. Reprod.*, **33**: 1213-1222, 1985.
5. Morris, I. D., Phillips, D. M., and Bardin, C. W. Ethylene dimethanesulfonate destroys Leydig cells in the rat testis. *Endocrinology*, **118**: 709-719, 1986.
6. Jackson, A. E., O'Leary, P. C., Ayers, M. M., and de Kretser, D. M. The effects of ethylene dimethane sulphonate (EDS) on rat Leydig cells; evidence to support a connective tissue origin of Leydig cells. *Biol. Reprod.*, **35**: 425-437, 1986.
7. Jackson, C. M., and Jackson, H. Comparative protective actions of gonadotrophins and testosterone against the antispermatogenic action of ethane dimethanesulphonate. *J. Reprod. Fertil.*, **71**: 393-401, 1984.
8. Forssmann, W. G., Ito, S., Weihe, E., Aoki, A., Dym, M., and Fawcett, D. W. An improved perfusion fixation method for the testis. *Anat. Rec.*, **188**: 307-314, 1977.
9. Turek, F. W., and Desjardins, C. Development of Leydig cell tumors and onset of changes in the reproductive and endocrine systems of aging F344 rats. *J. Natl. Cancer Inst.*, **63**: 969-975, 1979.
10. Rommerts, F. F. G., Grootenhuis, A. J., Hoogerbrugge, J. W., and van der Molen, H. J. Ethane dimethane sulphonate (EDS) specifically inhibits LH stimulated steroidogenesis in Leydig cells isolated from mature rats but not in cells from immature rats. *Mol. Cell. Endocrinol.*, **42**: 105-111, 1985.
11. Kerr, J. B., Risbridger, G. P., and de Kretser, D. M. Selective destruction of foetal Leydig cells of the neonatal rat testis; effects on testicular development, ultrastructure and function. 176th Meeting of the Society of Endocrinology, (Abstract 101). *J. Endocrinol.*, 1987.