Effect of Di-Butyl Phthalate on the Reproductive System of Male Rats

A Thesis

Presented to the Faculty of the Department of Biological Sciences of the State University of New York College at Brockport in Partial Fulfillment for the Degree of Master of Science

by

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THESIS DEFENSE

For Abhijit Adhyde

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Abstract

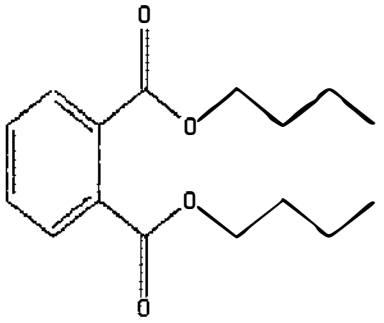
Reproductive malformations such as hypospadias, cryptorchidism and testicular cancer appear to be on the rise in certain human male populations. Declining sperm counts have also been reported in certain regions of the world, but these findings remain widely debated. Similar reproductive disorders have been seen in wildlife species and have been linked to highly contaminated ecosystems. An unifying mechanism was proposed to explain the decline in male reproductive health.[1] According to this hypothesis, all the alleged increases in male reproductive deficits in humans could be linked to disruption of critical developmental events occurring *in utero* and during the neonatal period when the fetal testes and particularly the Sertoli cells, are undergoing differentiation and replication. Thus, this theory centers on the disruption of the endocrine system during development by chemicals with hormone-like activities such as estrogens and anti-androgens.

The objective of this study was to determine the effects of endocrine disruptors on the reproductive system in male rats. In this study, Di-n-butyl phthalate (DBP), a known endocrine disruptor and a plasticizer was used. Male rats were fed with DBP for the duration of one month both at maturity (3 months) and when they were 1 month of age. After being fed the DPB for one month, some of the experimental animals were necropsied. A second objective was to extend this model to a continuous multi-generation breeding protocol, and to assess if the effects of DBP would be multiplied over the generations. The remaining rats of the original protocol were then bred with control female rats to obtain offsprings which would then be subjected to the same

feeding protocol. At necropsy, all animals were sacrificed to obtain serum levels for testosterone (T) measurement, and tissues of testes, accessory sex organs including prostate, seminal vesicles and epididymis were fixed for histological examination. The findings as observed in the first generation of treated rats were consistent with those reported in the literature for DBP. These include minor changes in occasional undescended testis, underdeveloped epididymis, prostate and/or seminal vesicles. However, these changes were observed to have amplified progressively in the successive generation of rats. In particular, the effects were much more pronounced when the feeding protocol were initiated in the immature (one month old) rats as compared to the sexually mature (3 months old) rats. The results clearly indicated that sexually immature rats at one month of age, even well passed the neonatal period, are more susceptible than the older rats at the time when DBP in food is administered; this period of susceptibility extends to the time the development of both the testes and the accessory sex organs are still being formed. Mature rats of the continuous multi-generation breeding protocol, when fed DPB, however, are by comparison quite resistant to the effects of DBP.

REVIEW OF LITERATURE

DBP, a phthalate ester with extensive world wide use in industry, is a colorless, oily liquid with a strong, bitter taste and a slight, aromatic odor.[2, 3] Dibutyl phthalate has a specific gravity of 1.0459 at 20° C and a boiling point of 340° C.[3] It is very soluble in acetone, benzene, alcohol and ether[4], but only slightly soluble in water.[5, 6]



Structural formula of DBP

DBP is used extensively in the manufacture of soft plastics (food packaging and cosmetics), medical supplies (transfusion and dental materials), textiles (as a lubricant and an insect repellant for impregnation in clothing)[7], and paper coatings.[8] Phthalate esters are known to leach from finished plastics into blood and milk and other foodstuffs and thereby enter the human body.[9] It has been estimated that 150 mg of DBP found in packing material will migrate into 1 kg of cheese with a 15 % fat content[10]. DBP likely derived from plastic tubes and bags has been identified in plasma and tissues of patients undergoing hemodialysis or receiving blood transfusions.[11]

Metabolism and Disposition

Metabolism and Disposition in Humans

As mentioned above, DBP has been detected in the in the blood of individuals via ingested food that was packed in containers lined with DBP.[9, 10] The concentration of DBP in the blood was much higher following ingestion of such food products (up to 0.35 ppm) than the average pre test value of 0.02 ppm. [12]

Metabolism and Disposition in Animals

After oral administration to rats and mice, DBP is thought to be metabolized by non-specific esterases in the gastrointestinal tract to mono- butyl phthalate (MBP) and butanol prior to absorption into the blood stream. [13] Metabolism, absorption and excretion in urine and feces are considered rapid; 31 to 44% of the administered dose of DBP was excreted in the urine and 20 to 22% was excreted in the feces 24 hours after administration. The maximum concentration of the metabolites in the plasma and various organs was reached approximately 20 to 30 mins after administration. Only traces of the parent compound, DBP, were found in the excreta. MBP was the major metabolite (70 to 80% of the total dose) and was mainly excreted in the urine. [7] Further hydrolysis of mono butyl phthalate to phthalic acid is thought to occur slowly. [14]

Toxicity

Dibutyl phthalate is of low to moderate acute toxicity in mammals. Dibutyl phthalate induces pleiotropic effects in rats and mice, including hypolipidemia, body weight gain, and often a marked hepatomegaly associated with hepatocellular replication, peroxisome proliferation, and induction or modulation of a variety of enzymes or enzymatic

pathways. [7]

Dibutyl phthalate has been shown to cause growth retardation in rats when administered in a diet at a concentration of 2,500 ppm for 1 year. [15] The pentobarbital sleeping times of male rats fed 1% Dibutyl phthalate for 26 days were decreased, presumably by induction of the cytochrome P_{450} system, compared with the sleeping times of rats fed only the basal diet. [16]

Reproductive Toxicity

Several important variables have been identified which modify testicular toxicity in phthalate-exposed rats, including the species, age of the animal and the dosing regimen. Oral administration of DBP consistently produces severe testicular atrophy in rats and guinea pigs, focal or no atrophy in mice and no testicular injury in hamsters.[17, 18] Young rats are more susceptible to phthalate induced testicular injury than adults. Four- week old rats exposed by gavage to 2.8gm/kg of di- (2-ethylhexyl) phthalate for 10 days show a decrease in testis weight and uniform atrophy of the seminiferous tubules while 10 week-old rats show normal testis weight and 5 to 50 % affected tubules; the testis of 15 week old rats are unaffected by this exposure.[19]

Lesions can be produced in adult rats by phthalate exposure; however the extent of injury is limited. [20]

The Sertoli cell has been identified as the primary cellular target for phthalate induced testicular injury by:

a. The early histopathological changes which occur in this cell type following both *in vivo* and *in vitro* exposure

b. The characteristic and early alterations in sertoli cell function and biochemistry, and

c. The rapid destruction of the sertoli-germ cell physical interaction. [21]

In addition, indirect mechanisms such as disruption of the pituitary-testes hormonal (FSH) axis [21] or urinary excretion and resultant depletion of testicular zinc [23] have been suggested.

In one study Mylchreest et al, [24] administered DBP to pregnant rats from gestational day 3 to post-natal day 20. At 750 mg/kg/day, the number of live pups per litter at birth was decreased and maternal effects on pregnancy and post implantation loss are likely to have occurred. Anogenital distance was decreased at birth in the male offspring at 500 and 750 mg/kg/day. The epididymis was absent or underdeveloped in 0, 9, 50 and 71% of the adult offspring (100 days old) at 0, 250, 500 and 750 mg/kg/day, respectively, and was associated with testicular atrophy and widespread germ loss. Hypospadia occurred in 0, 3, 21 and 43 % of the males. Ectopic or absent testes were seen in 0, 3, 6 and 29 % of males at 0, 250, 500 and 750 mg/kg/day. Absence of prostate gland and seminal vesicles as well as small testes and seminal vesicles were noted at low incidence in the 500 to 750-mg/kg/day-dose group. Dilated renal pelvis, frequently involving the right kidney, was observed in all DBP dose groups. Vaginal opening and estrous cycles were not affected in the female offspring, although, low incidences of reproductive tract malformations, mainly involving the development of uterus, were observed in two and one rat at the 500 and 750 mg/kg/day doses, respectively. All the exposed groups showed adverse effects on the male reproductive tract structure and indices of puberty. Based on this study, the LOAEL (Lowest Observed Adverse Effects Level) was 250 mg/kg/day.

Based on the relationship between testes weight / histopathology and sperm production, the relationships between sperm numbers and fertility[24], and the number of major malformations of the reproductive tract, it is expected that at least the high and mid- dose animals would be sub-fertile.

In one study, oral exposure to di n-butyl phthalate of male rats for up to 34 days at doses up to 2500 mg/kg/day resulted in decreased testicular weight, atrophy of seminiferous tubules and decreased sperm count [25, 26, 27]

Mechanism:

What could be the mechanism of endocrine disruption of DBP? The focus of attention is on these endocrine active chemicals that may alter male sexual differentiation through their anti androgenic activity. [28]

Androgens, the male steroid hormones, are essential at all stages of reproductive life. The two primary androgens are testosterone and its reduced metabolite dihydrotestosterone (DHT). Both bind to the androgen receptor (AR) in target cells and initiate expression of androgen-responsive genes. In the fetus, androgen-mediated signaling pathways are crucial for masculinization of the male fetus. The testes play an important role in this process.[29] See fig. 1 for details.

Testosterone production by the Leydig cells of the fetal testes begins at gestational day 15. Testosterone plays an important role in the differentiation of the Wolffian ducts into epididymis, vas deferens, ejaculatory duct, and seminal vesicles. Differentiation of these structures occurs near the time of birth in a male rat and is mediated by locally produced DHT from testicular testosterone by the enzyme 5-α reductase. Hypospadias or cleft phallus occurs as a result of lack of fusion of the genital folds that envelop the penile

urethra. DBP elicits many of the antiandrogenic effects of an AR antagonist; however some of its effects are not consistent with such a mechanism. In one such study, a competitive binding assay was done and both DBP and MBP, its major metabolite did not inhibit binding of androgen to AR.[28] Thus DBP does not appear to induce its antiandrogenic effects by AR antagonism, but, it may act by an unique mechanism. [30] Thus DBP may exert its antiandrogenicity by indirectly interfering with androgen signaling pathways during sexual differentiation, possibly by acting on the fetal testes. (See fig. 2)

Studies on early fetal effects of DBP have indicated that on gestation day 18, morphological changes can be observed in the testes, characterized by an apparent proliferation of Leydig cells, with multinuclear gonocytes found in seminiferous cords. This study also indicated that fetal testicular testosterone was also significantly reduced on GD 18 and 21, and that the Leydig cells undergoing hyperplasia were both AR and 3-ß hydroxy steroid dehydrogenase-positive by immunocytochemistry. AR localization appeared decreased in the ducts of the developing epididymis. Taken together, these changes would suggest that a primary androgen insufficiency coupled with a decreased expression of AR is responsible for the failure of the epididymis to develop to develop in DBP treated fetuses. Immunostaining for the proliferating cell nuclear antigen (PCNA) indicated normal presence in the fetal Sertoli cells, but the Leydig cells were not immunopositive. Moreover, the gonocytes in control fetuses exhibited very limited staining, whereas those from DBP treated animals were markedly immunoreactive. These data thus suggest that the presence of gonocytes in DBP treated fetal testes were due to an inappropriate initiation of cell division, whilst the observed increase in Leydig

cell number may be due to a compensatory mechanism as a result of lowered fetal testicular testosterone. [31] Thus, the epididymis is most sensitive to the induction of malformations[32] and the initial event appears to be a decrease in the fetal testicular testosterone. Studies in older animals exposed to phthalate esters have indicated that the Sertoli cell is the likely target [33]

One study suggests that testicular effects of Di butyl phthalate may be associated with the effects of di butyl phthalate on zinc metabolism. [13, 18] Zinc is an essential element for the development of testes and administration of di butyl phthalate increases the urinary excretion of zinc and decreases the zinc content of the testes. Administration of zinc to young male rats given 1000 mg/kg/day of di butyl phthalate showed substantial protection against the testicular injury produced by di butyl phthalate. [13]

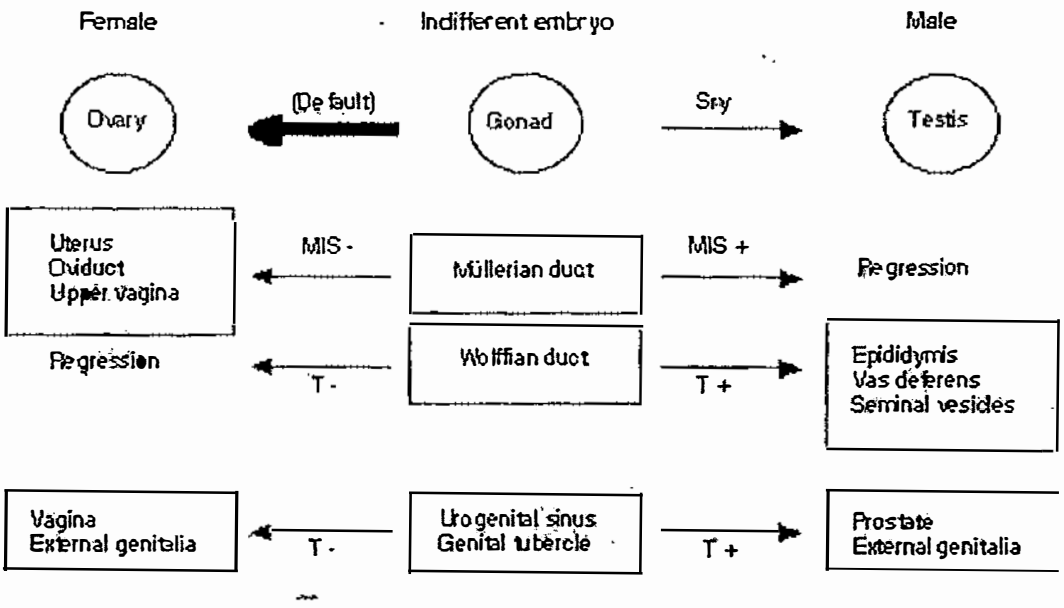
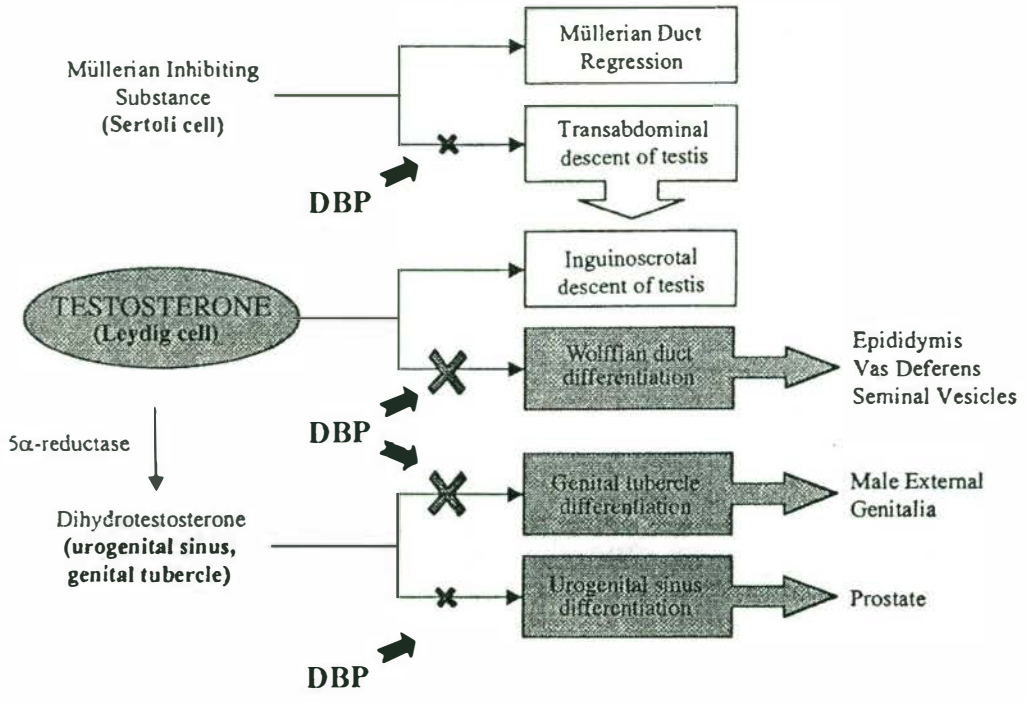


Fig. 1

Sexual differentiation in mammals involves development of the female or the male reproductive organs from undifferentiated structures during embryonic and fetal life. Mullerian inhibiting substance (MIS) produced by Sertoli cells in the fetal testes is required for the regression of the Mullerian ducts, which would otherwise develop into female reproductive organs. Testicular testosterone, which is produced by fetal Leydig cells, is essential for differentiation of the urogenital sinus and genital tubercle through dihydrotestosterone. Blocking of MIS or testosterone signaling results in abnormal development of the male reproductive tract.



Schematic representation of effects of Di Butyl Phthalate on male reproductive system of rats.

MATERIALS AND METHODS

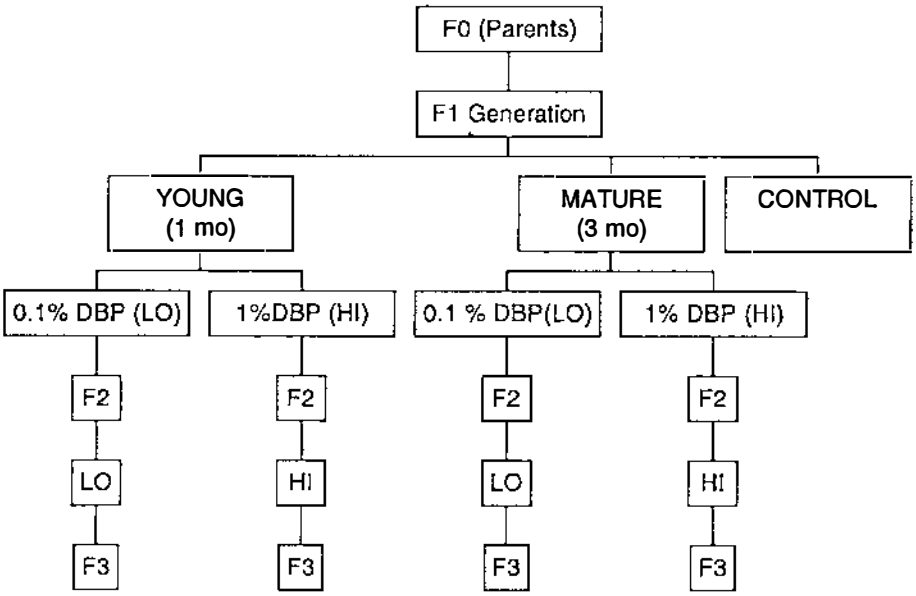
This is a prospective multigenerational study of the effects of DBP on the reproductive system of male rats. Male Fischer (F 344) rats were studied and followed from the first generation to the fourth generation. The rats were bought from Charles River Lab. DBP, purchased from Sigma Lab had a specific density of 1.04g/ml. The rats were initially fed with rat pellets. The rat powder 'chow' (AIN-93M) that was purchased from Dyets Inc. was the medium through which the rats were fed DBP. The DBP was mixed manually with the rat chow. Being an oily liquid, it became miscible after thorough mixing. Two concentrations of DBP, 0.1 % and 1 % were fed to two experimental groups, while the control rats were fed chow with 0 % DBP. The table below explains the rationale of the concentration of DBP in food.

	1 % DBP	0.1 % DBP	CONTROL
DBP	30 ml	3 ml	0 ml
OIL	270 ml	297 ml	300 ml
FOOD	3000 gm	3000 gm	3000 gm

It was calculated that each rat consumed approximately 15 g diet /day. DBP with corn oil was added as 10% V/W to the rat food.

The rats were housed in cages in the animal room that was maintained according to the requirement of the relevant authorities. The rats , called the F0 generation were 30 days old at receipt and on reaching sexual maturity, they were mated with the female rats. These F0 rats are not fed DBP. The resultant litter (F1 generation) was divided into

control and 0.1 % and 1 % rats. Six (6) rats were included in each group and they were fed DBP at the concentrations of 0.1 % and 1 % for duration of one month. The chart below explains the protocol that was followed to feed the rats.



A cohort of the rats that were obtained from the mating of the original (F_0) generation was fed DBP when they were sexually immature and another cohort was fed DBP when they were sexually mature. The male rats become sexually mature at two months. They were fed DBP at the age of three months. DBP was fed to both the cohorts for one month. Other rats were mated with control female rats and thus the next generation of rats was born. The offspring of the rats that were fed a particular diet treatment were fed the same diet, i.e. the offspring of a rat that is fed 1 % DBP at 1-month age will be fed the same DBP dosage diet at the same age. This allows us to categorize the effects observed in the different treatment through multiple generations. A group of rats was also fed with

control diet for comparison purpose.

Necropsy:

Some of the rats were sacrificed by decapitation to collect trunk blood. Necropsy was performed to find out the histological changes in the testes and other reproductive organs. Liver and kidney were also removed for gross pathological changes

The animals were weighed before the necropsies and the animals were killed by decapitation using a guillotine. Trunk blood was obtained at that time. The blood was allowed to clot and then centrifuged. The serum was separated and stored at – 20 °C. The epididymis was exposed, a small incision was done on the epididymis and 1µl of epididymal sperms were aspirated using a micropipette. The sperm count was done using a Neubar's chamber at the dilution of 1:10. The seminal vesicles and the prostate were dissected. The weights of testes, epididymis, prostate and seminal vesicles were noted. Apart from the reproductive organs, the weights of liver and kidney were also noted.

Radioimmunoassay:

Radioimmunoassay (RIA) was carried out using the protocol of Chan *et al.*[37] The isotope that was used for the RIA was 1, 2, 6, 7- ^{3}H- Testosterone from New England Nuclear Lab. The antiserum to testosterone was also obtained from New England Nuclear Biomedical laboratories. It was prepared by Dr. Stephen Chan and has been previously characterized by Chan *et al.* Standards were used to obtain the standard curve for both the hormones. The hormones were extracted from the serum samples as follows. The extraction was carried out by adding 150 µl of deionized water to 50µl of serum. To this 2 ml of ether was added and the resultant was kept in the freezer for 15 min. The organic

portion was vortexed. The extraction was repeated and the organic phases were pooled and evaporated to dryness under N_2 gas. Nitrogen gas was diffused through this solution till it dried off completely. Absolute alcohol was added and N_2 gas was diffused through it again till it dried of completely.

Before commencing with the RIA, titer tests were performed that resulted in approximately 50% binding of the isotope, which is considered ideal for the experiment. In this case the titer used for the experiment was 1:3000 of BTS-1 for Testosterone and 1: 1500 of AES-1 for Estradiol. The following table summarizes how the procedure was performed.

	Antiserum (0.1ml)	Tracer (0.1ml)	Buffer (ml)
Total count	0	* * *	0.2
Charcoal Blank	0	* * *	0.2
Serum Samples	* * *	* * *	0.1
Standards	* * *	* * *	0.1
Quality controls	* * *	* * *	0.1

0 = not added
*** = Amount added

All the assays were performed in duplicates.

Immunohistochemistry:

Inhibin was used as an antigen to demonstrate the effects of DBP on the testis. Inhibin is localized in the sertoli cells and acts as its marker. The aim of using inhibin immunohistochemistry was to demonstrate that there is a disruption of the blood testis barrier of Sertoli cells. The ABC kit, purchased from the Vector labs, Burlingame, CA

was used. The principle of the ABC (**A**vidin- **B**iotin- horseradish peroxidase macromolecular **C**omplex) is that avidin has an extraordinary affinity for the small molecular weight vitamin called Biotin. Thus the 2-° antibody was conjugated to biotin and is called as the biotinylated antibody. Then, a preformed Avidin Biotin horseradish peroxidase macromolecular reagent was added (ABC reagent) to amplify the reaction and produce a more translucent reaction. Then, diaminobenzidine tetrahydrochloride (DAB) was used as a horseradish peroxidase substrate to enable us to view the staining on the tissue. This ABC kit comprised of a secondary (2 °) antibody that was biotinylated, ABC reagent, and the blocking serum. The 1° antibody was mouse anti human Inhibin antibody, 2° antibody was horse anti mouse biotinylated antibody and horse-blocking serum was used to block the extra binding sites. The antigen retrieval was done prior to proceeding with the immunohistochemistry protocol. A Black and Decker steamer that reached temperatures of 95 ° C was used. The slides were immersed in the antigen retrieval solution (PBS buffer) at 95 ° C for 10-15 minutes. There were, however, some technical problems with the antigen retrieval and instead frozen sections were cut of the testis and immunostaining was done using the same protocol without using antigen retrieval. The protocol for immunostaining is as follows.

1 5 to 8 μ thick sections were obtained from the wax embedded tissue and sections were fixed on slides coated with poly lysine.
2 The tissues were deparaffinized and dehydrated with xylene and graded alcohols.
3 Steamer was used for antigen retrieval.
4 The sections were incubated in a humidified chamber for 30 mins with Horse blocking serum.

5 Sections were washed with the buffer

6 Sections were incubated in 1° antibody (mouse anti inhibin) for 30 min.

7 They were again washed with buffer

8 They were then incubated with 2° biotinylated antibody (horse anti-mouse) for 30 min.

9 Sections were washed with the buffer

10 Incubation with ABC reagent was carried for 30 min.

11 They were again washed with the buffer

12 DAB was used to stain the slides till the staining intensity develops.

13 Hematoxylin and Eosin was used for counterstaining

Since there were technical difficulties in demonstrating staining using immunohistochemistry, the sections were also stained by Hematoxylin and Eosin. The protocol used for that is attached in the appendix.

RESULTS

NECROPSY RESULTS:

Significant differences were observed between the body weights of control and F1Ylo, and F2YHi, F2YLo and F3YLo generation of rats. Table 1 highlights the body weights of rats in the groups of rats fed with different diets. There appears to be no significant differences in the body weights of the control rats and F1YHI. There appears to be significant differences between the body weights of Control and F2YHI, F2YLO and F3YLO animals. Table 1 also lists the weights of the reproductive organs including testis, epididymis, seminal vesicles and prostate. The weight difference between the control animals and F1YHI appears to be significant in the testis and seminal vesicles. The epididymis and the prostate don't appear to be influenced in this group of animals. In the F1YLOgroup of animals, there is a significant difference in the weights of testis, seminal vesicles and prostate. The mean weight of the epididymis in control was 0.04 gm more than the F1YLO, however this difference was not found to be significant. For the F1MHI group of rats, except for the prostate, the weights of the other reproductive organs were found to be significantly different from the control group. In the F2YHI group of rats, there were significant differences observed in the weights of testis, seminal vesicles and prostate, with the p values being 0.01, 0.003 and 0.03 respectively. In the F2Ylo group, the differences in weights observed in the testis, epididymis and prostate with the controls were significant. Similarly, in the F3Ylo group, the differences were tested by the student's t test and the differences obtained were found to be significant.

The sperm count was measured and it is listed in Table 1 and the respective p values are

listed in Table 2. There appears to be significant decrease in the sperm count of F1YHi and F2YHi rats when compared to control. The difference observed in the sperm counts in the control and F1YLo does not appear to be significant.

HISTOLOGY AND IMMUNOHISTOCHEMISTRY:

There appears to be a definite change in the structure of the testis as seen in the different groups of rats. Figure 1 illustrates a normal testis with well-preserved seminiferous tubules and interstitium. In F1YHi rats, there is some vacuolation in the seminiferous tubule and appearance of some inflammatory infiltrate in the interstitium. However, in the F2YHi rat testis, seen in Fig. 3, more definitive changes are seen. There appears to be separation of the germinal epithelium from the basement membrane of the seminiferous tubule and some of the spermatids appear to have pycnotic nuclei. In the F3MLo generation of rats, seen in Fig. 4, there appears to be a relative degeneration of the architecture of the seminiferous tubule in the testis. There appears to be some cell debris in the interstitium, the origin of which could be from the seminiferous tubule or from the interstitium itself. Fig. 5 of F3YHi demonstrates severe disruption of the architecture of the seminiferous tubules with appearance of spaces inside the tubule and cell debris inside the interstitium. Immunostaining of the testis for Inhibin was attempted and fresh frozen section of the testis yielded some positive stain, with no stain observed in the negative control and a strong positive stain in the positive control slide of spleen. This is illustrated in Fig. 6. Thus, the differences observed were maximally seen in the F2 and the F3 generations. The effects were more pronounced in the young rats compared to the mature rats and in the F3 generation compared to the F2 generation.

RADIOIMMUNOASSAY:

The levels of serum testosterone obtained in the various groups of rats are seen in table 3. The significance of these results is attached as table 4. There appears to be significant differences in the levels of serum testosterone between control rats and rats fed with DBP, the exception being the rats of F1YLO and F1MLO generation. Table 5 indicates that statistically significant differences were observed in the F1YHi and F1MHi groups and the F2YLo and F2MLo groups.

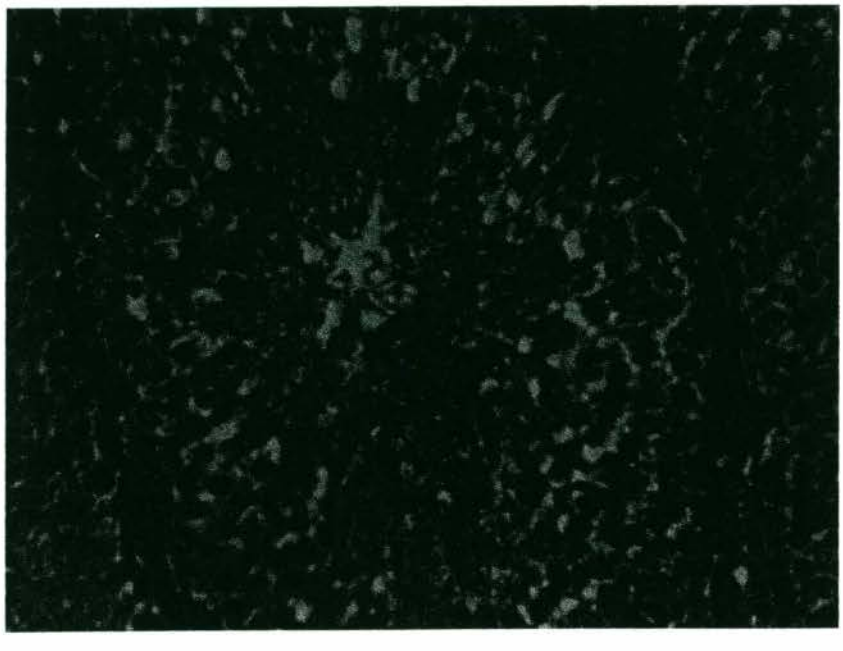
The standard curve in Appendix II was one of the curves used for obtaining the levels of serum testosterone.

OTHER OBSERVATIONS:

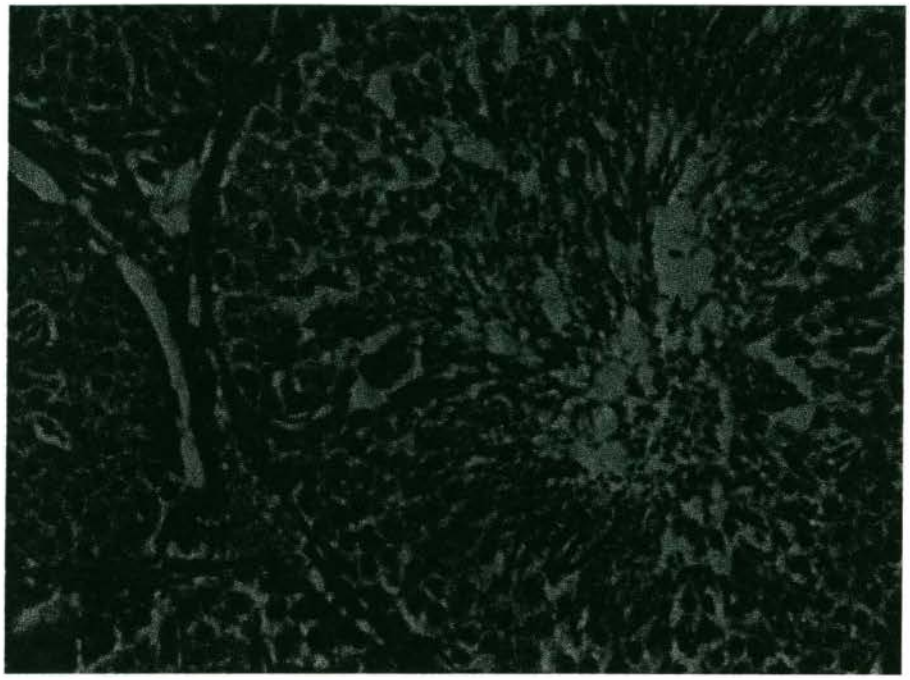
The number of live pups per litter delivered by the female control rats mated with DBP fed animals was also observed to be fewer in number compared to the control animals. Also, the weights of the pups delivered by the rats that were mated with DBP fed male rats was generally observed to be less than the weight of the pups delivered by control male rats. The fecundity in rats that were fed with DBP was found to be less than control animals. The time taken to impregnate the normal female rats was observed to be more in the DBP fed rats compared to the controls in this study. However, we do not have the precise statistics for this study. Two rats in the F2MLO generation had undescended testis on one side. Few rats in the F3Yhi had testes that were very soft and fluid in consistency with under developed epididymis.

Control Testis showing normal architecture

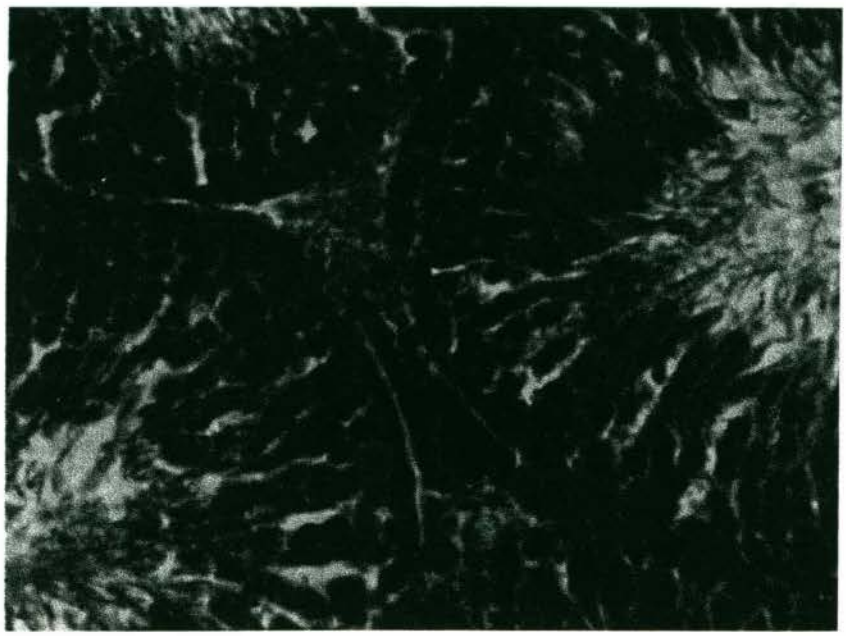
F1YHI testis showing vacuolation



F2YHI showing inflammatory infiltrate in interstitium



F3MLO



F3YHI showing degeneration of the seminiferous tubule

Immunostaining with Inhibin showing staining in interstitium

Table 1 showing the body weights and weights of reproductive organs

	STATUS	BODY WT. (gm)	TESTIS (gm)	EPIDIDYMIS (gm)	SEMINAL VESICLES (gm)	PROSTATE (gm)	SPERM COUNT * 10^6/ml
CTRL		357	3.02	0.82	0.51	0.51	40.6
		316	2.95	0.83	0.37	0.23	43.6
		298	2.93	0.75	0.50	0.42	32.9
		321	2.9	0.75	0.42	0.50	41.2
		376	3.33	0.97	0.46	0.29	36.5
		360	3.14	0.93	0.45	0.28	43.5
		383	3.05	0.92	0.50	0.39	41.8
		367	3.27	0.98	0.42	0.27	
Mean ± S.D		**347± 31.3**	**3.07± 0.15**	**0.86 ± 0.09**	**0.45 ± 0.04**	**0.36 ± 0.10**	**40.01 ± 3.9**
	HI	343	2.7	0.78	0.22	0.42	32.6
		347	3.01	0.88	0.44	0.39	33.6
		344	2.82	0.76	0.40	0.26	29.8
		344	2.83	0.81	0.42	0.23	31.5
		337	2.98	0.81	0.48	0.29	33.6
		344	2.70	0.78	0.2	0.41	35.5
		366	3.01	0.88	0.44	0.39	
	Mean ± S.D	**346 ± 9.14**	**2.86 ± 0.13**	**0.81 ± 0.04**	**0.37 ± 0.11**	**0.34 ± 0.07**	**32.7 ± 1.9**
	LO	310	2.98	0.84	0.48	0.30	38.5
		310	2.97	0.83	0.54	0.42	41.6
		302	2.67	0.88	0.47	0.30	39.5
		281	2.55	0.85	0.55	0.18	35.6
		324	2.63	0.79	0.50	0.24	42.5
		293	2.45	0.76	0.46	0.15	41.5
	Mean ± S.D	**303 ± 14.9**	**2.7 ± 0.21**	**0.82 ± 0.04**	**0.50 ± 0.03**	**0.26 ± 0.09**	**39.7 ± 2.5**
	HI	287	3.05	0.79	0.46	0.17	
		373	3.04	0.88	0.41	0.22	
	Mean ± S.D	**330 ± 60.8**	**3.045 ± 0.007**	**0.83 ± 0.06**	**0.43 ± 0.03**	**0.19 ± 0.03**	
	LO	338	2.9	0.79	0.48	0.20	
		356	3.06	0.88	0.51	0.32	
		348	2.95	0.90	0.44	0.32	
		390	2.85	0.85	0.58	0.27	
	Mean ± S.D	**358 ± 22.5**	**2.94 ± 0.08**	**0.85 ± 0.04**	**0.50 ± 0.05**	**0.27 ± 0.05**	

Table 1 continued

		HI	298 307 193 155	2.13 2.89 3.15 2.35	0.72 0.84 0.53 0.54	0.55 0.53 0.53 0.54	0.27 0.34 0.19 0.15	21.5 26.5
		Mean ± S.D	**238 ± 75.8**	**2.63 ± 0.47**	**0.65 ± 0.14**	**0.53 ± 0.09**	**0.23 ± 0.08**	**24 ± 3.5**
		LO	272 286 292 286	2.85 2.64 2.88 2.77	0.77 0.74 0.80 0.77	0.39 0.43 0.41 0.53	0.16 0.26 0.28 0.29	
		Mean ± S.D	**284 ± 8.4**	**2.78 ± 0.10**	**0.77 ± 0.02**	**0.44 ± 0.08**	**0.24 ± 0.05**	
		HI	301	2.8	0.65	0.36	0.16	
		LO	286	2.83	0.78	0.38	0.22	
		HI						
		LO	280 247	2.76 2.53	0.77 0.72	0.40 0.37	0.26 0.16	
		Mean ± S.D	**263 ± 23.3**	**2.64 ± 0.16**	**0.74 ± 0.03**	**0.33 ± 0.02**	**0.21 ± 0.07**	
		HI						
		LO	293 284	2.68 2.75	0.76 0.72	0.37 0.44	0.24 0.29	

Table 2: Significances of differences in the groups of rats in the above-mentioned parameters. *P< 0.05 = significant difference, calculated by student's t test.

GROUPS OF ANIMALS		BODY WEIGHT	TESTES	EPIDIDYMIS	SEMINAL VESICLE	PROSTATE	SPERM COUNT
	F1YHI	**0.47**	0.009	**0.09**	0.04	**0.34**	0.009
	F1YLO	0.04	0.018	**0.15**	0.039	0.05	**0.45**
	F1MHI	**0.28**	**0.40**	**0.32**	**0.31**	0.03	
	F1MLO	**0.27**	**0.07**	**0.39**	**0.07**	**0.09**	
	F2YHI	0.002	0.01	**0.052**	0.0039	0.03	0.007
	F2YLO	0.001	0.004	0.03	**0.34**	0.04	
	F3YLO	0.04	0.004	0.05	0.04	0.05	

Table 3: Serum Testosterone (ng/ml)

	CTRL	F1YLo	F1YHi	F1MLo	F1MHi	F2YLo	F2YHi	F2MLo	F2MHi	F3YLO	F3YHI	F3MLO	F3MHI
	5.80	5.27	4.90	5.10	4.70	4.59	4.40	5.00	4.30	4.10	3.98	4.20	3.62
	5.90	5.65	5.22	5.90	4.80	4.50	4.50	4.80	4.25	3.98	3.85	4.35	3.65
	5.70	5.95	5.25	5.59	5.02	4.30	4.70	5.15	4.50	4.26	3.40	4.25	3.59
	5.80	5.70	5.40	5.59	5.25	4.42	4.80	4.97	4.18	4.38	3.65	3.87	3.54
	5.50	5.85	5.22	5.51	4.80	4.62	4.60	5.10	4.35	3.87	3.81	3.97	3.48
	5.38	5.60	5.23	5.80	5.00	4.20	4.39	4.96	4.41	3.95	3.55	3.94	3.99
Mean +/- S.D	5.68+/- 0.2	5.67+/- 0.235	5.20+/- 0.164	5.58+/- 0.278	4.93+/- 0.201	4.44+/- 0.165	4.57+/- 0.165	5.00+/- 0.122	4.33+/- 0.114	4.09+/- 0.197	3.71+/- 0.213	4.10+/- 0.195	3.65+/- 0.179

Table 4 showing the significances of serum testosterone in different cohorts. $P < 0.05$ is considered statistically significant.

CTRL	F1YLO	NS*
CTRL	F1YHI	P=0.0011
CTRL	F1MLO	NS
CTRL	F1MHI	P<0.0001
CTRL	F2YLO	P<0.001
CTRL	F2YHI	P<0.0001
CTRL	F2MLO	P<0.0001
CTRL	F2MHI	P<0.0001
CTRL	F3YLO	P<0.0001
CTRL	F3YHI	P<0.0001
CTRL	F3MLO	P<0.0001
CTRL	F3MHI	P<0.0001

Table 5 indicating the significance of differences in serum testosterone levels in young and mature rats.

F1YHi	F1MHi	p = 0.0133
F1YLo	F1MLo	p=0.28
F2YHi	F2MHi	p=0.08
F2YLo	F2MLo	p<0.0001
F3YHi	F3MHi	p=0.29
F3YLo	F3MLo	p=0.47

DISCUSSION

Di-n-butyl phthalate, a plasticizer with potential for human exposure, was confirmed to have effects on the reproductive system of male F 344 rats. This study differed from the other studies done previously in that it was a multigenerational study, unlike previous studies that were carried out in just one or two generation. This study was also the first to use Inhibin as a stain to demonstrate the breach of the blood testes barrier. Inhibin has not been used in previous studies to demonstrate this. This is the first time that effects of feeding DBP have been carried into successive generations indicating that DBP has an impact on the DNA of the rats. As observed in the previous literature on DBP, there was a decrease in the body weight of the rats that were fed with the experimental diet compared to the control diet. According to this study, the decrease in the body weight is also observed in the 2nd and 3rd generation of rats. Previous studies have shown that young, pubertal rats are more sensitive than sexually mature animals to testicular toxicity.[34] This study concurs with previous studies that show that the DBP affects younger rats more than mature rats. This is evident because there is no significant change in the weight of mature rats of the 1st and 2nd generations. The reason for reduction in body weight could be due to decrease appetite of rats; it was observed throughout the study that rats fed with DBP consumed less food than the rats on control diet. The exact measure of this difference in food intake is not known. However, several other factors could be implicated,[7] including albumin and protein loss in renal or intestinal disease, impaired protein synthesis, increased catabolism and poor nutritional status due to a combination of factors described above. There is no consistent difference, however in the serum levels

of testosterone in the young and mature rats.

Testicular toxicity of DBP was confirmed in this study. Morphological indicators of toxicity, namely epithelial degeneration was observed. The changes were more profound in the animals that were young (1 month) and received high doses of DBP. The histological changes observed in the testes include separation of the germinal epithelium from the basement membrane. The 2nd generation of rats (F2YHI) and 3rd generation (F3YHI) showed disorganization of the seminiferous tubules. The spermatogenesis was disturbed. The tubular lumen had clear irregular spaces devoid of sperms. The interstitium was sometimes filled with cell debris. There was also evidence of disorganization of the interstitium. These changes are also reflected in the sperm counts that are significantly decreased in the 1st and 2nd generation of rats fed with high dose of DBP at 1-month age. Some seminiferous tubules were irregular in shape and size. Some of the tubules showed evidence of sloughing off of spermatogenic layers. In one of the slides that showed some positive immunostain from Inhibin, it was demonstrated that Inhibin was positive in the interstitium and the seminiferous tubule both. In could be possible that there is a breach in the sertoli cell barrier and the Inhibin made in the sertoli cell has leaked in the interstitium. However, there needs to be more study done by comparing it with controls.

Dibutyl Phthalate also affects the reproductive system in male rats by altering the serum levels of Testosterone. The changes observed in serum testosterone along with the other observations like change in body weight and histopathological changes suggest that DBP impacts the Sertoli cell and the interstium. Previous studies have noted either a decreased concentrations of serum testosterone [35] or unchanged levels compared to control. [36] In this

study, the levels of serum testosterone were significantly reduced in animals that were fed with DBP in a manner that is amplified in this multi generation study. These changes were carried forward to the next generation and were maximally seen in the F3 generation, suggesting that the effect of DBP is cumulative and probably multiplies over the next generation.

Drawbacks of this study:

i. Some of the data in this study was lost.

ii. Estrogen evaluations were technically difficult and inaccurate due to clustering of multiple values over a small range of the graph.

iii. A lack of primary antibody specific to rats against Inhibin and other sertoli cell markers. Problems also encountered in antigen retrieval that could not be resolved. This led to use of frozen sections with some staining which could not be done on rats of F2 and F3 generation.

iv. There were problems encountered in breeding animals of the desired groups whose data was lost. The breeding of these animals resulted in pups that were eaten by their mothers. It needs to be further investigated whether DBP was indeed responsible for this altered behavior.

CONCLUSION

Although the exact mechanism by which Dibutyl Phthalate causes testicular injury is not yet understood, it is considered to have definite effects on the reproductive system of male rats. All the parts of this study suggest that Dibutyl phthalate affects the male reproductive system in various ways:

i. Morphological changes in the testes and its ultrastructure.
ii. Decrease in the serum levels of reproductive hormones, namely Testosterone
iii. Decrease in the body weight and weight of the reproductive organs like Testis, epididymis, prostate and seminal vesicles
iv. DBP thus impacts the seminiferous tubule and the interstitium. The Leydig cell production of testosterone is decreased in a manner that is amplified in the next generations.

Further research:

A satisfactory molecular explanation of the mechanism of phthalate-induced testicular injury must account for the known unique aspects of this injury:

i. A greater susceptibility of young rather than old animals
ii. A greater susceptibility of certain stages of seminiferous tubule epithelium
iii. Histopathological changes like vacuolation and alteration of the blood testis barrier
iv. Occurrence of an inflammatory infiltrate in the interstitium

APPENDIX

Appendix I: Reagents and protocol

PBS:

Dissolve 1 tablet in 200 ml of Deionized water to obtain:

0.01 M Phosphate buffer

0.0027 M Potassium chloride and

0.137 M Sodium chloride, pH 7.4

DAB – Urea $H_2 O_2$ solution:

1 tablet of DAB and 1 tablet of Urea- $H_2 O_2$ dissolved in 10 ml of Deionized water

Hematoxylin and Eosin staining protocol:

Deparaffinize for 2 mins. in Xylene, 3 changes each.

Rehydrate in absolute and then 95 % alcohol followed by three changes of distilled water.

30 sec. in Lillie Mayer's hematoxylin

10 seconds in Sodium Borate

Wash in 3 changes of distilled water followed by 30 seconds in Absolute alcohol

30 seconds in Working Eosin solution.

Dehydrate in graded alcohols and xylene and then mount with a mounting media like permount.

Working solution of Eosin:

100 ml Eosin stock solution

300 ml 80% Alcohol

1ml Acetic acid to each 100 ml of stain just before it is ready for use.

PBS-0.1 % gel:

NaCl: 17.33 gm

Na_2HPO_4 anhydrous: 17.33 gm

NaH_2PO_4 H_2O: 10.8 gm

Na azide: 2.0 gm

Gelatin: 2 gm

H_2O: 2 lit.

Adjust pH to 7.0 ± 0.05

Protocols for standard and antiserum dilution:

Standard:

Stock = 10 mg/100ml i.e. 10,000 ng/0.1ml

Dilute 10µl stock to 10 ml (10 ng/0.1ml) [A]

1 ml (A) 10 ml (1 ng/0.1 ml) [B]

1 ml (B) 10ml (100 pg/0.1 ml)

0.5 ml (B) 10 ml (50 pg/0.1 ml)

0.5 ml (A) 10 ml (500 pg/0.1ml)

250 µl (A) 10 ml (10 pg/0.1 ml)

1 ml (100 pg/0/1 ml) 10 ml (10 pg/0.1 ml)

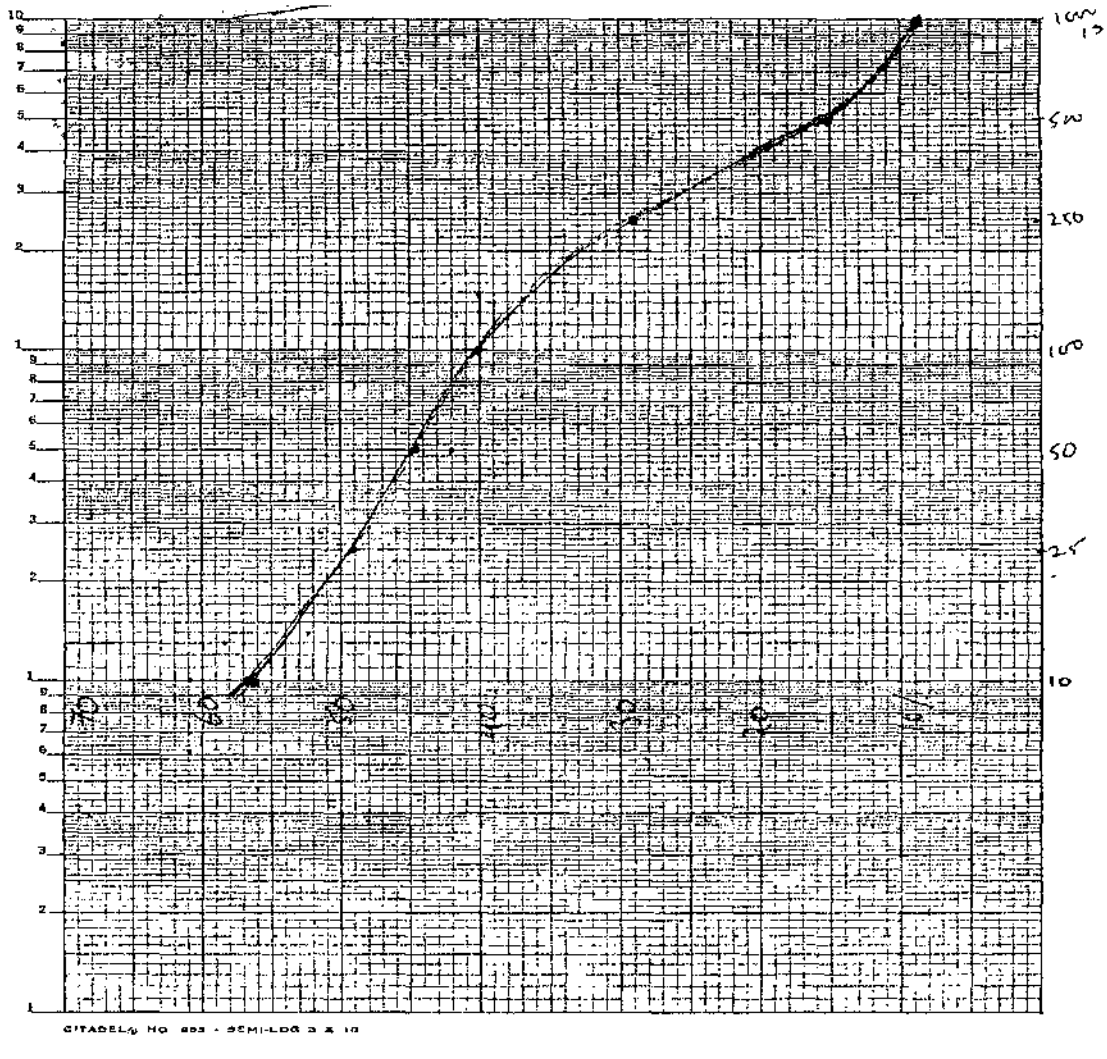
1 ml (250 pg/0.1 ml) 10 ml (25 pg/0.1 ml)

Antiserum

100 µl stock, dilute to 1000 µl (10 x)

100 µl (10x)	1000 µl (100 x) [A]
200 µl (A)	1000 µl (500 x)
100 µl (A)	1000 µl (1000 x)
100 µl (A)	1500µl (1500 x)
50 µl (A)	1000µl (2000 x)
20 µl (A)	1000 µl (5000 x)
10 µl (A)	1000 µl (10,000 x)
5 µl (A)	1000 µl (20,000 x)

Appendix II: Standard curve



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