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Title: AN IN VIVO STUDY OF CADMIUM-INDUCED CHROMOSOMAL CHANGES IN SOMATIC AND GERMINAL TISSUES OF C57B1/6J MALE MICE

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James C. Hampton

For the first time the in vivo mutagenic potential of cadmium chloride was evaluated by chromosomal examination of both bone marrow cells and spermatocytes of C57B1/6J mice. An acute exposure to cadmium was administered in a single subcutaneous injection. The doses were 0.0252, 0.0126, 0.0055 and 0.0000 mmoles cadmium/kg body weight followed by sacrifice at 6, 24, and 48 hours. Mice were also given a subacute exposure with the same doses split into five injections which were administered at 24 hour intervals. Animals were sacrificed 6 hours after the last injection. Bone marrow cells from mice receiving an acute and split dose subacute exposure were examined for chromosomal damage by scoring 50 metaphase plates. To assess the mutagenic potential of cadmium through a complete cycle of spermatogenesis and to examine the extended exposure response of bone marrow cells, an experiment

with an extended duration of acute exposure (30 days) was carried out with doses of 0.0252 and 0.0000 mmoles cadmium/kg body weight.

Organ weight/body weight ratios and histologic evaluation of liver, kidney, spleen and testis were procedures used to monitor the occurrence and extent of cadmium damage in the animals evaluated for mutagenic events. Differential leucocyte counts, reticulocyte counts and total bone marrow cell counts were used to evaluate marrow damage.

In the bone marrow cells of acutely exposed mice, chromatid breaks and deletions increased in frequency within 6 hours. After subacute exposure breaks, deletion and despiralizations were present in increased frequencies. With extended acute exposure, rearrangement and pulverization were noted in addition to breaks and despiralization.

Spermatocytes exposed to 0.0252 mmole cadmium/kg body weight had increased frequency of achromatic gaps, breaks, autosomal univalents and stickiness. Positive correlation of gaps and breaks from bone marrow and spermatocyte were noted while negative correlations for rearrangements and stickiness were found when data were pooled by treatment. Bone marrow cells consistently had more aberrations than spermatocytes. When the correlations of aberrations between the tissue of individual animals were examined no significant correlations were found.

Organ weight/body weight ratios and histologic examination of liver, kidney, spleen, and testis revealed severe damage to the liver occurred within 24 hours and persisted through day 30. The spleen responded with proliferation of white pulp by day 3. This proliferation also persisted through day 30.

Assessment of bone marrow response to cadmium differential leucocyte counts, reticulocyte counts and total bone marrow cell counts revealed erythropoietic stress with no marked influence on the white cell populations.

The mutagenic, histologic and pathological effects noted in this study were reviewed in relation to previous cadmium chromosome aberration and mutagenesis literature.

An *in vivo* Study of Cadmium-induced Chromosomal Changes in Somatic and Germinal Tissues of C57B1/6J Male Mice

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Typed by Mary Jo Stratton for Theresa Lynn Felten

What we have learnt, is like a handful
of Earth,

While what we have yet to learn, is like
the whole World.

Poetess Saint Avvaiyar

as translated by S.S. Iyer

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AN IN VIVO STUDY OF CADMIUM-INDUCED CHROMOSOMAL CHANGES IN SOMATIC AND GERMINAL TISSUES OF C57B1/6J MALE MICE

I. INTRODUCTION

Increased cadmium exposure at all levels of the ecosystem due to increased industrial use of metals makes it imperative that in vivo mutagenic properties of cadmium be better characterized. The objectives of this study were:

1. To determine whether cadmium at maximum tolerated and at occupational or normal exposure doses resulted in chromosomal aberrations in C57B1/6J male mice.
2. To determine whether chromosomal aberrations would be detectable simultaneously in somatic and germinal tissues and whether these aberrations were correlated.
3. To determine minimum exposure parameters required for significant chromosomal change.

The direct chromosome preparation of bone marrow cells (Tjio and Whang, 1965) was employed in order that any selection factors inherent in in vitro lymphocyte or bone marrow cultures could be circumvented. In that way a more accurate determination of the mutagenic properties of cadmium in intact somatic tissue was possible. In germ cell examination more subtle chromosomal changes were sought in order to determine more accurately the

heritable mutagenic potential of cadmium. Observation of dividing spermatocytes gave the maximum chance of ascertaining chromosome changes before selection eliminates aberrant cells (Leonard, 1973).

Review of Literature

The toxic properties of the environmental contaminant cadmium were first noted in 1858 (Fulkerson and Goeller, 1973). However, only recently has there been any concern over the nature and severity of its effects. The occurrence, physical and chemical properties, metabolism, toxicity and associated pathology of cadmium have been reviewed by Fassett (1975), Fishbein (1976), Flick, Kraybill and Dimitroff (1971), Friberg, Piscator, Nordberg and Kjellstrom (1974), Fulkerson and Goeller (1973), Gunn and Gould (1970), Hiatt and Huff (1975), Huisingh (1974), Neathery and Miller (1975), Nobbs and Pearce (1976), Nordberg (1974), Pier (1975), Schubert (1973), Vigliani (1969), and Wood (1976). A review of experimental evidence indicates that cadmium compounds qualify as toxic substances according to most criteria of the National Institute for Occupational Safety and Health (Christensen, 1974; see Appendix I). However, present experimental results are in conflict regarding the mutagenic properties of cadmium compounds.

Several studies indicate that cadmium reacts with DNA, binding preferentially to repeated DNA sequences in chromosomes (Mizuhira and Kimura, 1973; Sissoeff, Grisvard and Guille, 1975) with both phosphates and bases serving as potential binding sites (Hise and Fulkerson, 1973; Goodgame, Jeeves, Reynolds and Skapski, 1975a, 1975b; Clark and Orbell, 1975). Goodgame et al. (1975) indicate that it is possible that the metal ions could bind adjacent purines in nucleic acids causing inhibition of replication.

There is substantial evidence that certain other metals and metal ions can cause chromosomal damage (Muro and Goyer, 1969; Nishioka, 1975; Paton and Allison, 1972; Skerfving, Hansson and Lindsten, 1970; von Rosen, 1953). One could expect that toxic cadmium compounds would also disrupt chromosomes, producing genetically abnormal cells. However, present findings are not consistent concerning cadmium mutagenesis.

The first study supporting this mutagenic hypothesis (von Rosen, 1953) tested a halogenic series of metals for their ability to complex with and to break chromosomes of Pisum rootlet cells. Cadmium was found to form strong complexes with the chromosomes and was a potent chromosome breaker. Despiralization occurred in prophase and there was some indication of mutagenic selectivity in causing chromosome breaks. Noncomplex-forming elements of this study had no radiomimetic activity.

Oehlkers (1953), in a report concerning chemically-induced chromosome breaks in Vicia, cited unpublished work by Glass which indicated that cadmium nitrate preferentially broke satellite regions of the Vicia chromosomes. Mutagenic activity was optimum at M/10,000 with higher concentrations being less effective mutagens. No interpretation of this phenomenon was offered.

Friberg et al. (1974) and Fulkerson and Goeller (1973) both cite the contradicting unpublished studies of Ramel and K. Friberg in which cadmium chloride fed to Drosophila melanogaster larvae had no mutagenic effect. In the first study the frequency of sex chromosome loss was determined as an indication of chromosome breakage. In this study no significant chromosome loss occurred. A second experiment, consisting of exposure to cadmium chloride combined with 3,000R X-irradiation, was performed to observe cadmium effects on sex-linked recessive lethals. No significant increase in recessive lethals due to cadmium was noted nor a significant change in chromosome repair mechanisms.

Unpublished work by Grosch (cited by Fulkerson and Goeller, 1973) indicated that cadmium acetate fed to females of the parasitic wasp Habrobracon at near-lethal doses reduced reproductive capacity but mutations could not be detected. It was suggested that it may not be possible to alter DNA at cadmium doses that would not also be lethal.

Using the rec-assay, Nishioka (1975) determined cadmium chloride to be a DNA-damaging agent whereas cadmium nitrate was not. In this assay procedure wild type strains of Bacillus subtilis and rec-mutants, lacking repair enzymes, were tested with cadmium and 54 other potential mutagenic metal compounds. Increased DNA damage was detected by differences between the zones of inhibition for wild type and rec- cells exposed to paper discs saturated with a particular compound.

Mammalian mutagenic studies involving cadmium compounds have taken several approaches. Shiraishi, Kurahashi and Yosida (1972) determined that cadmium sulfide caused chromosome aberrations in in vitro-treated human lymphocytes. However, Paton and Allison (1972) were unable to find significant aberration induction with cadmium chloride. In the same year Shiraishi and Yosida (1972) reported a very high frequency of chromosome abnormalities in the cultured lymphocytes of "Itai Itai" disease patients (i.e. those Japanese accidentally poisoned by food and water supplies contaminated with cadmium). More recently Bui, Lindsten and Nordberg (1975) were unable to obtain similar results, leaving doubtful the in vivo effect of cadmium in humans. Doyle, Pfander, Crenshaw, and Snethen (1973) reported extreme hypodiploidy in in vivo-exposed sheep leucocytes. In their study rams were fed 60 μg cadmium/g of

food for 191 days, blood samples were collected on day 191 and the leucocytes cultured for 72 hours before chromosome analysis.

Industrial workers with occupational exposure to cadmium and cadmium and lead combined have also been studied for aberrant cultured lymphocyte chromosomes. Bui et al. (1975) found no significant difference between cadmium-exposed workers and controls. It should be noted that no attention was given to exposure to other metals in selecting workers and controls. Deknudt and Leonard (1975) and Bauchinger, Schmid, Einbrodt and Dresp (1976) found high yields of severe chromosome abnormalities in zinc smelting plant workers exposed to cadmium and lead. Although these investigators were unable to demonstrate a causal relationship between cadmium and aberrations, the possibility of synergistic effects of combined metals could not be excluded.

In tissue culture studies conflicting results have also been reported. In a test system of human diploid cell strains W138 and MRC5, Paton and Allison (1972) found cadmium chloride to have no significant effect on chromosome continuity, although they did note more frequent over-constriction of the chromosomes. Rohr and Bauchinger (1976) found cadmium sulfate treatment of Chinese hamster cells produced a significant number of chromatid type aberrations, i.e., single breaks and exchanges, as well as an increased number of gaps. The cadmium concentrations of this

study more closely approximate the higher doses known to occur in humans than the doses in the Paton and Allison study.

Dominant lethal assays with cadmium chloride using mice have been done in three separate instances (Epstein, Arnold, Andrea, Bass and Bishop, 1972; Suter, 1975; Gilliavod and Leonard, 1975) and in all three studies the results were negative. Epstein et al. (1972), however, point out that the dominant lethal assay is only one indicator and dominant lethal inactive agents cannot be regarded as nonmutagenic until in vivo cytogenetic and host mediated assay procedures have also been tested. Leonard (1973) also stated that control values in dominant lethal tests exhibited great variation thus limiting the resolution of the test.

Two germinal cell aberration studies involving cadmium have been reported to date. Gilliavod and Leonard (1975) examined spermatocytes of BALB/c mice for chromosomal rearrangements and found no translocation figures. In their examination no other chromosomal aberration observations were made. Shimada, Watanabe and Endo (1976) sought to determine the mutagenicity of cadmium in the mouse oocytes. Meiotic spread plates in metaphase II were examined for chromosomal abnormalities. A dose of 6 mg cadmium/kg body weight was capable of producing a statistically significant increase in numerical chromosome anomalies. Structural anomalies were not observed.

II. MATERIALS AND METHODS

Animals

C57B1/6J male mice obtained from Jackson Harbor Laboratory (Bar Harbor, Maine) were maintained in the laboratory for a minimum of 14 days prior to use. Animals had free access to food (Wayne Lab Blox, Allied Mills, Chicago, Illinois) and deionized-distilled water. At the time of experimentation, the ages of the young males ranged from 12 to 16 weeks. The average weight was 26.1 g. No animal less than 20 g was considered for experimental use. Mice were randomly assigned to treatment and control groups and housed individually in 29.8 by 18.3 cm plastic cages with crushed corn cob bedding and stainless steel wire cage covers.

Treatment Protocol

Dosage Determination

Initial determination of cadmium chloride dosages to be used was based on evidence from the literature that 0.101 mmoles cadmium/kg or 0.0505 mmoles cadmium/kg was the maximum dose tolerated by mice in general (Gunn, Gould and Anderson, 1968). However, C57B1/6J males, chosen for this study because they are not susceptible to rapid cadmium-induced testicular necrosis and

therefore are one of the few strains that can be examined for genetic damage (Gunn and Gould, 1970), have been reported by Taylor, Heiniger and Meier (1973) to be more susceptible to acute cadmium toxicity than purebred susceptible lines or hybrids. It was necessary, because of this, to determine a maximum dosage for subsequent experiments.

A rough approximation of the C57B1/6J strain tolerance to cadmium was obtained from four groups of eight male mice, randomly established and earmarked for identification purposes. A 0.2 ml injection of cadmium chloride in sterile distilled water was administered subcutaneously in doses of 0.101, 0.0505, 0.0252, or 0.0126 mmoles cadmium/kg body weight. The general appearance, eating and drinking habits, and body movements of surviving animals were observed and mortality recorded every six hours for the first four days and then on a daily basis for three weeks. The maximum dose chosen was that level at which more than 50% of the animals survived the three-week observation period.

The minimum level (0.0055 mmole cadmium/kg body weight) used was based on the average total body burden at middle age for humans (Fulkerson and Goeller, 1973). An intermediate concentration, between the maximum and normal exposure level, was chosen for the third treatment level. A 0.2 ml subcutaneous injection of deionized-distilled water was given as a negative control.

Mutagenic Testing Procedure

Chromosome methodology for mutation testing has been recommended by the Ad Hoc Committee of the Environmental Mutagen Society (Nichols, Moorhead and Brewen, 1972).

For the acute study the experiment was conducted as a 3 x 4 factorial with three levels of time of sacrifice (6 hours, 24 hours, 48 hours) and four levels of cadmium dose (0.0252, 0.0126, 0.0055, 0.0000 mmole cadmium/kg body weight). Five mice were assigned at random to each time x dose combination, and 50 metaphase plates were scored for each animal (see Appendix II).

For the subacute study the experiment was conducted with four levels of cadmium dose (0.0252, 0.0126, 0.0055, 0.0000 mmole cadmium/kg body weight). Each cadmium dose was divided for five equal split-dose injections administered at 24 hour intervals. Mice were sacrificed 6 hours after the last injection. Five mice were assigned at random to each dosage and 50 metaphase plates were scored for each animal (see Appendix II). Testing in this study adhered to those recommendations as regards animal type and age, tissue examined, testing scheme of the compound, and scoring of aberrations. Deviations from the recommended procedures were as follows: additional control animals were included, injections were given subcutaneously rather than intraperitoneally, and positive controls, i.e., one producing chromosomal aberrations without

metabolism and one producing chromosomal aberrations with metabolism, were not included. Additionally, a duration of exposure experiment was carried out to determine the simultaneous mutagenic effects of cadmium chloride on bone marrow cells and spermatocytes. For the exposure duration study the experiment was conducted as a 4 x 2 factorial with four levels of sacrifice (day 1, day 3, day 7, day 30) and two levels of cadmium dose (0.0252, 0.0000 mmole cadmium/kg body weight). Four mice were assigned at random to each time x dose combination and 50 metaphase and 50 diakinesis plates were scored for each animal (Appendix II). Pretreatment body weight and a blood sample obtained by tail clipping for a differential white blood cell count were taken prior to injecting each animal. These same measures were taken at time of sacrifice.

At the time of sacrifice, a code number was assigned to each animal. To prevent biased interpretation this number rather than dose and time was used on the bone marrow processed for chromosome examination according to the Tjio and Whang (1965) procedure (see Appendix III), on the right testis, prepared for spermatocyte examination according to the Meredith procedure (1969; see Appendix IV), and on the liver, kidney, spleen, and testis taken from each animal for histological examination. Organ weights were recorded for liver, left kidney, spleen and left testis and these organs were fixed in McDowell-Trump's fixative (1976).

Staining and Histological Procedures

Cell suspensions from bone marrow and testis were prepared and were stained with Wolbach Giemsa stain (Carolina Biological Supply Co., Gladstone, OR) according to the modified procedure of Lillie (1965; see Appendix V).

After fixation the liver, spleen, left kidney and left testis were dehydrated and embedded in methacrylate (see Appendix VI). Sections 3 μ thick were cut on a JB-4 microtome (Sorvall). These sections were floated onto glass slides and allowed to dry. When thoroughly dry, they were stained with hematoxylin and eosin (see Appendix VII) and a coverslip was applied.

Chromosomal Analysis

The entire surface of bone marrow and spermatocyte slides was scanned because of the tendency of the fixative to spread the cells. A Zeiss Universal Photomicroscope equipped with a Plan 16X objective lens was used for this scanning. When metaphase or diakinesis plates were found they were photographed at a total magnification of 225x using a Neofluar 63X oil immersion objective. Photographs were taken of each spread for evaluation of chromosome number, achromatic gaps (G), deletions (D), exchanges (R), breaks (B), and "other" aberrations, e.g., pulverization (P), stickiness (S), despiralization (DS) and XY and autosomal univalents (XU and AU) in

the spermatocytes. Precedent for including the evaluation of univalents comes from the recommendations of Leonard (1973) and Tates and Natarajan (1976). Although gaps were recorded, they were not included in the determination of the total number of cells with one or more abnormalities. The reasons for this were that it has not been ascertained whether the gaps represent some loss of chromosomal material and that it has been shown that gaps can be repaired in subsequent cell divisions (Brinkley and Hittelman, 1975). The conventional distinction between gaps and breaks, based on alignment and strand continuity (Nichols, Moorhead and Brown, 1973; Cohen and Hirshborn, 1971) were used in this study. Additionally, measurements of the length of the normal strand was compared to the damaged strand. If a broken strand is measured, it would be of equal length without the inclusion of the intervening space. A strand with a gap would only be of equal length if the space is included (B. McClanahan, personal communication). According to the testing protocol a total of 50 metaphase (diakinesis) plates were to be examined for each animal. Poor staining and spreading of plates sometimes made it impossible to obtain the necessary number of usable plates, but a minimum of 30 was always examined. When all photographs had been evaluated, code numbers were translated and treatment and control animal data were grouped for statistical analysis.

Photography

Photography was completed with a Zeiss Universal photomicroscope equipped with a 35 mm camera base and with conventional and phase optics. All chromosome preparations were photographed on Kodak High Contrast Copy Film 5069 using a Neofluar 63X oil immersion objective and a 0.12 neutral density filter. Film was developed 4.5 minutes in Kodak D-19 developer, rinsed and agitated 30 seconds, fixed 2 minutes in Kodak Rapid Fixer, washed 20 minutes in running water and treated in Kodak Photo-Flo Solution for 30 seconds. Prints were made on Afga-Gevaert single weight, contrast 4 paper.

Differential Leucocyte Counts, Bone Marrow Total Cell Counts and Reticulocyte Counts

In order to determine if the bone marrow cell populations were altered in any way during cadmium treatment, two pilot tests were done. Prior to treatment two drops of blood were collected by tail clipping for two pretreatment differential leucocyte count slides. Ten male mice were then injected with 0.0252 mmoles cadmium/kg body weight cadmium chloride in a 0.2 ml injection. Ten male mice injected with 0.2 ml sterile deionized-distilled water served as control animals. All 20 mice were maintained for 30 days. Post injection differential leucocyte counts were done using tail clip blood

from each animal on days 1, 3, 7, and 30. These counts would indicate changes in the circulating blood cell populations and indirectly indicate changes in the marrow populations. It would also be possible, by the presence of Howell-Jolly bodies and polychromatic staining of red blood cells, to determine if the erythropoietic system of the marrow was being stressed.

Prior to sacrifice on day 30, a 0.2 ml unheparinized blood sample was collected by cardiac puncture for a reticulocyte count. A significant increase of reticulocytes in peripheral blood would indicate specific bone marrow stress of the erythropoietic system. Animals were sacrificed by cervical dislocation and the right femur was removed for a bone marrow total cell count. The femur was cleaned of all muscle and connective tissue, the distal end removed, and 1 ml of Isoton, an isotonic saline, was injected into the shaft at the trochanteric fossa to remove all marrow cells. Care was taken to insure even removal of the distal end from each femur and to flush each femur completely. Three 20 lambda samples of this marrow suspension were counted using a Coulter Counter, Model Z_B (aperture current = 0.354, amplification = 1/2, and threshold = 15) and a mean value determined.

Differential leucocyte counts were stained with Wright's Giemsa stain. Reticulocyte slides were stained by mixing 10 drops of blood with 10 drops of cresyl violet and staining for 10 minutes. A drop of

the blood/stain mixture was applied to a clean glass slide and a thin film spread by drawing out the drop using a second slide. The slides were air dried and then stained with Wright's Giemsa.

All blood slides and marrow samples for the total cell count were coded. Blood slides were read by a medical technologist who was unaware of the treatments given.

Statistical Procedures

Prior to statistical analysis of chromosome aberration data, an arc sine transformation was performed to stabilize the variances, since the number of cells examined per animal differed. The statistical tests used on the aberration data were then performed on the transformed proportion of affected cells to the total cells analyzed rather than directly on the number of affected cells per animal.

Analysis of both cytogenetic and organ weight data was completed by computer with the Statistical Package for the Social Sciences (SPSS) using the Northwestern University version.

Initially the total number of aberrant cells, the total number of aneuploids, and the total number of structural aberrations per total cells examined for each animal were analyzed using two-way analysis of variance (ANOVA) procedures. Tests on these groupings would determine if any extensive mutagenic effects were present. The subsequent analyses would determine more specifically if any significant individual aberrant effects had occurred.

Two-way ANOVA procedures were used to analyze aneuploids, gaps, breaks, deletions, rearrangements, despiralization, stickiness, and pulverization scored in the acute bone marrow study. The three levels of significance considered in these analyses and all subsequent statistical procedures were $P < 0.01$, $P < 0.05$, and $P < 0.10$. The $P < 0.10$ level was included because this less statistically significant probability level was still very likely to indicate biologically significant effects. The subacute experiment was analyzed separately. There was no strong justification for comparing these data with those obtained in the acute exposure experiment since there was a significant time difference.

Statistical analysis of experimental data related to exposure duration was done in three phases. Bone marrow and spermatocyte aberrations were each analyzed with two-way ANOVA procedures. The data were then combined for regression analysis. In correlating bone marrow and spermatocyte data, groupings by treatment, by time, and by treatment and time were analyzed.

Organ weights, expressed as proportions of the individual animal total weights, were compared with two-way ANOVA procedures.

F-testing was applied to the mean values of the differential leucocyte counts and to the means of the bone marrow total cell count and of the reticulocyte count.

III. RESULTS

Dosage Determination

The cadmium chloride dose determined as the maximum tolerated (MTD) by C57B1/6J males was 0.0252 mmoles cadmium/kg body weight. As can be seen from Table 1, of those injected with 0.0252 mmoles cadmium/kg body weight only one mouse died on day one and none of those injected with 0.0126 mmoles died. This maximum tolerated dose was used with the first acutely treated animals and all survived. No subsequent mortality was noted with this maximum dose. All animals receiving this dose did manifest behavioral and pathological changes associated with acute cadmium poisoning.

Among the classical cadmium poisoning changes observed with the higher than maximum and maximum tolerated doses during the 21 days post injection were sluggish movements, hunched postures, coarse, ruffled hair and rapid shallow respiration. Animals at these higher doses had injection-site swelling which appeared with 24 hours and persisted 48 to 96 hours. No injection-site swelling occurred in animals treated with 0.0126 mmoles cadmium/kg body weight.

Autopsies done on animals dying in the first four days revealed expected poisoning associated focal lesions of the liver, discoloration

of the kidneys, and enlargement of the spleen. The gut was also severely affected. The stomach failed to empty, the small intestine was dilated and the intestinal wall was thin and translucent.

Table 1. Results of a 21-day examination of cadmium chloride tolerance by C57B1/6J male mice after a single subcutaneous injection.

Cadmium dose administered (mmoles/kg)	Number of dead mice/total mice injected				
	Day 1	Day 2	Day 3	Day 4	Day 21
0.1010	6/8	8/8	8/8	8/8	8/8
0.0505	1/8	2/8	4/8	5/8	5/8
0.0252	1/8	1/8	1/8	1/8	1/8
0.0126	0/8	0/8	0/8	0/8	0/8

Mutagenic Testing

Acute-Subacute Exposure Experiment

Tables 2, 3 and 4 summarize the results of the analysis of the grouped total aneuploids (TAN), total structural changes (STR) and total number of abnormal cells (TAB) as a proportion of the total cell examined. A very significant increase ($P < 0.02$) in structural aberrations with time was noted. However, total structural aberrations were not significantly affected by cadmium dose. Neither total aneuploids nor the total number of abnormal cells showed significant effects over time or with dose. These initial groupings and analyses

Table 2. Initial chromosomal analysis from acute cadmium exposure of bone marrow cells. Results expressed as the proportion [total number of abnormal cells/total number of cells examined].

Cadmium dose	Proportion of abnormal bone marrow cells			
	Time of sacrifice			
(mmoles/kg)	6 hours	24 hours	48 hours	Mean
0.0252	0.346	0.520	0.469	0.445
0.0126	0.532	0.494	0.478	0.501
0.0055	0.540	0.560	0.559	0.553
0.0000	0.483	0.527	0.657	0.555
Mean	0.475	0.525	0.540	0.513

S.E. = 0.014

Table 3. Initial chromosomal analysis from acute cadmium exposure of bone marrow cells. Results expressed as the proportion [total number of aneuploid cells/total number of cells examined].

Cadmium dose	Proportion of aneuploid bone marrow cells			
	Time of sacrifice			
(mmoles/kg)	6 hours	24 hours	48 hours	Mean
0.0252	0.331	0.443	0.355	0.376
0.0126	0.489	0.428	0.431	0.449
0.0055	0.439	0.471	0.454	0.455
0.0000	0.447	0.449	0.605	0.500
Mean	0.426	0.447	0.461	0.445

S.E. = 0.019

Table 4. Initial chromosomal analysis from acute cadmium exposure of bone marrow cells. Results expressed as the proportion [total number of structurally aberrant cells/total number of cells examined].

Cadmium dose (mmoles/kg)	Proportion of structurally aberrant bone marrow cells			
	Time of sacrifice			
	6 hours	24 hours	48 hours	Mean
0.0252	0.103	0.298	0.433	0.278
0.0126	0.239	0.194	0.215	0.216
0.0055	0.065	0.284	0.341	0.230
0.0000	0.104	0.183	0.227	0.171
Mean**	0.127	0.239	0.304	0.225

S.E. = 0.011

**Significant at the 5% level.

may have combined abnormalities which cannot be justifiably considered similar, e.g., breaks and stickiness, or may also have concealed one or two significant aberrant features. The very significant structural aberration group also required further analysis to determine specifically which forms of structural changes were most prevalent.

Individual aberrations found in bone marrow cells are listed in Table 5. From the two-way ANOVA it was determined that breaks were the only aberrations occurring in significant numbers (Time $P < 0.1$; Dose $P < 0.01$) in bone marrow cells receiving short-term acute exposure in vivo. For representative photographs of a normal mitotic figure and of a figure with a break, see Figures 1 and 2.

Chromosome aberration effects of subacute exposure to cadmium chloride are presented in Table 6. Subacute exposure resulted in significant dose-related occurrences of breaks and despiralization. In the case of differences among deletions, the significance did not arise from a dose-dependent effect. For representative photographs of despiralization and deletion, see Figures 3 and 4.

Exposure-Duration Experiment

Bone Marrow Aberration. When exposure to cadmium in vivo at MTD was extended to include day 7 and day 30, significant

Table 5. Evaluation of specific chromosome aberrations after acute cadmium exposure of bone marrow cells.

Cadmium dose (mmoles/kg)	Time of sacrifice 6 hours	24 hours	48 hours	Mean
A. Proportion of aneuploids - Acute exposure				
0.0252	0.271	0.228	0.251	0.250
0.0126	0.353	0.244	0.194	0.264
0.0055	0.359	0.274	0.208	0.280
0.0000	0.312	0.336	0.476	0.375
Mean	0.324	0.271	0.282	0.292
S.E. = 0.027				
B. Proportion of gaps - Acute exposure				
0.0252	0.054	0.105	0.113	0.091
0.0126	0.044	0.079	0.056	0.059
0.0055	0.052	0.095	0.126	0.091
0.0000	0.039	0.063	0.085	0.062
Mean	0.047	0.085	0.095	0.072
S.E. = 0.015				
C. Proportion of deletions - Acute exposure				
0.0252	0.015	0.029	0.022	0.022
0.0126	0.020	0.013	0.018	0.017
0.0055	0.020	0.021	0.028	0.023
0.0000	0.013	0.011	0.045	0.023
Mean	0.017	0.019	0.028	0.021
S.E. = 0.002				
D. Proportion of breaks - Acute exposure				
0.0252	0.048	0.104	0.196	0.116**
0.0126	0.023	0.104	0.116	0.081**
0.0055	0.029	0.129	0.151	0.103**
0.0000	0.013	0.046	0.093	0.051**
Mean*	0.028	0.095	0.139	0.087
S.E. = 0.005				

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Table 5. (Continued)

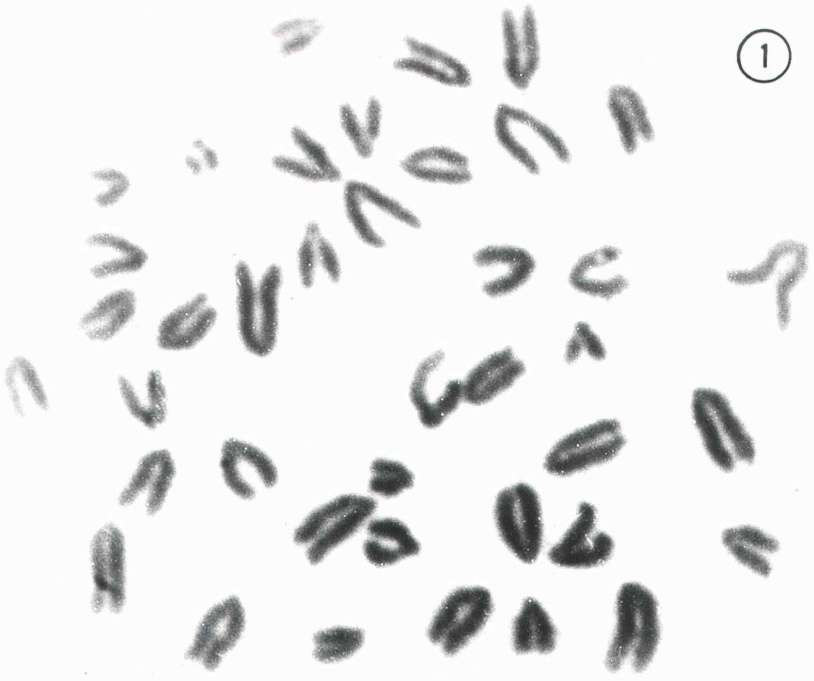
Cadmium dose (mmoles/kg)	Time of sacrifice 6 hours	24 hours	48 hours	Mean
E. Proportion of rearrangements - Acute exposure				
0.0252	0.015	0.013	0.051	0.021
0.0126	0.020	0.013	0.013	0.015
0.0055	0.025	0.021	0.019	0.021
0.0000	0.018	0.015	0.020	0.017
Mean	0.019	0.015	0.025	0.019
S.E. = 0.003				
F. Proportion of stickiness				
0.0252	0.012	0.038	0.022	0.023
0.0126	0.018	0.013	0.023	0.018
0.0055	0.020	0.047	0.029	0.032
0.0000	0.045	0.032	0.032	0.036
Mean	0.023	0.032	0.026	0.026
S.E. = 0.003				
G. Proportion of despiralizations - Acute exposure				
0.0252	0.025	0.023	0.013	0.020
0.0126	0.025	0.026	0.018	0.023
0.0055	0.023	0.021	0.024	0.022
0.0000	0.023	0.025	0.020	0.023
Mean	0.024	0.024	0.018	0.022
S.E. = 0.002				
H. Proportion of pulverizations - Acute exposure				
0.0252	0.019	0.016	0.019	0.018
0.0126	0.020	0.015	0.019	0.018
0.0055	0.020	0.020	0.024	0.021
0.0000	0.013	0.011	0.020	0.015
Mean	0.018	0.015	0.021	0.018
S.E. = 0.001				

*Significant at the 10% level.

***Significant at the 1% level.

Figure 1. Normal mouse bone marrow mitotic figure. 2250X.

Figure 2. Mouse bone marrow mitotic figure with a chromatid break (lower arrow) and two terminal chromatid gaps (upper arrows). 2725X.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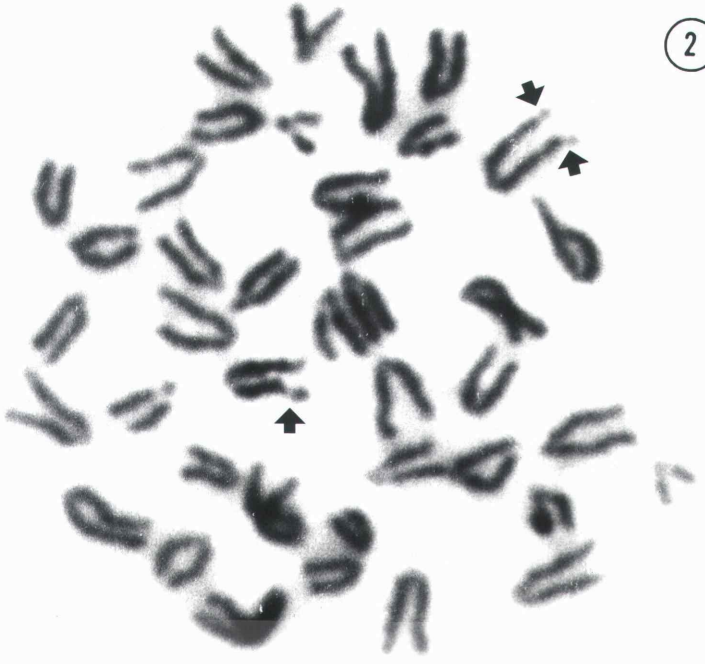
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Table 6. Evaluation of specific chromosome aberrations after sub-acute cadmium exposure of bone marrow cells.

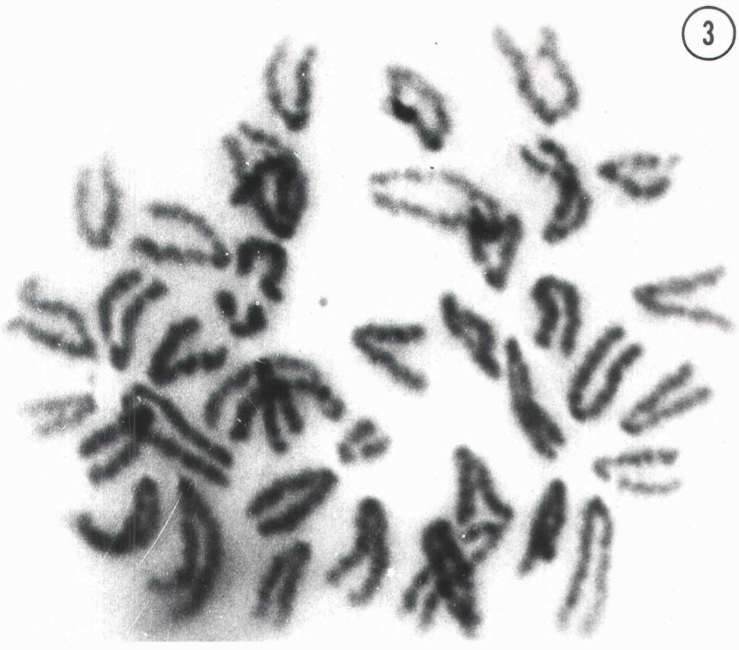
	Cadmium dose			
	0.0252	0.0126	0.0055	0.0000
	A. Proportion of aneuploids			
Mean S.E. = 0.030	0.170	0.451	0.346	0.261
	B. Proportion of gaps			
Mean S.E. = 0.015	0.094	0.039	0.153	0.115
	C. Proportion of deletions			
Mean*** S.E. = 0.006	0.025	0.034	0.043	0.023
	D. Proportion of breaks			
Mean*** S.E. = 0.003	0.063	0.058	0.031	0.032
	E. Proportion of rearrangements			
Mean S.E. = 0.002	0.034	0.039	0.016	0.032
	F. Proportion of stickiness			
Mean S.E. = 0.002	0.077	0.038	0.039	0.049
	G. Proportion of despiralization			
Mean** S.E. = 0.002	0.031	0.039	0.038	0.024
	H. Proportion of pulverization			
Mean S.E. = 0.002	0.031	0.039	0.036	0.040

** Significant at the 5% level.

*** Significant at the 1% level.

Figure 3. Mouse bone marrow mitotic figure that has become despiralized. 2475X.

Figure 4. Mouse bone marrow mitotic figure with a terminal deletion (arrow). 2250X.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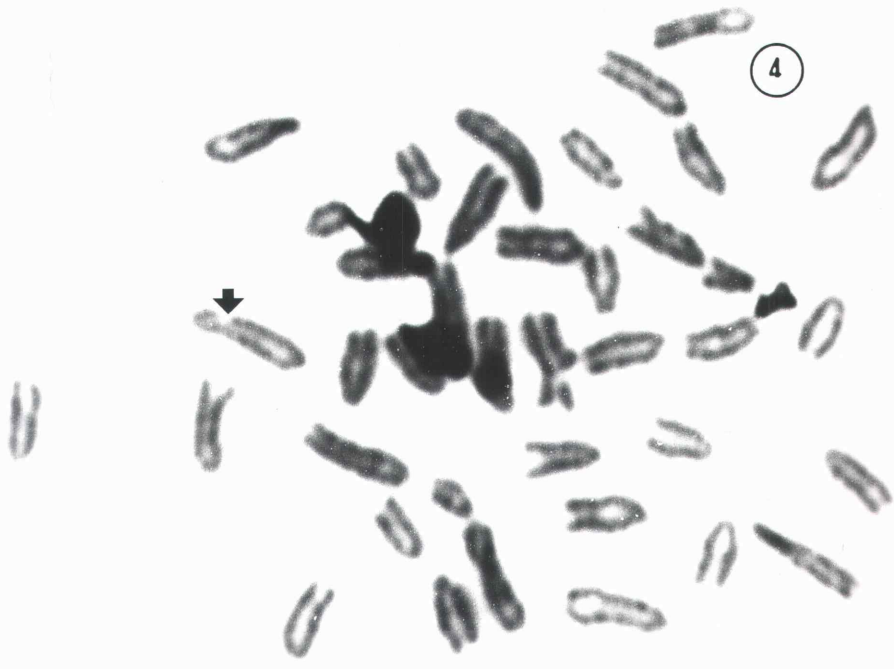
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increases in the number of bone marrow aneuploids ($P < 0.01$), breaks ($P < 0.01$), rearrangements ($P < 0.05$) and pulverizations ($P < 0.05$) occurred in a time-related fashion (see Table 7). Dose-related changes in the number of breaks ($P < 0.05$), rearrangements ($P < 0.1$), pulverizations ($P < 0.10$) and despiralizations ($P < 0.05$) were also significant. With further analysis the significant increase in the number of aneuploids was determined the result of a very significant dose-by-time interaction. Figure 5 is a representative photograph of one form of chromosomal rearrangement, the dicentric. Figure 6 is a representative photograph of an aneuploid and Figure 7 is a representative photograph of pulverization.

Spermatocyte Aberrations. To summarize the results of spermatocyte aberration analysis recorded in Table 8, gaps ($P < 0.1$), breaks ($P < 0.01$), rearrangements ($P < 0.01$) and stickiness ($P < 0.05$) differ significantly with time. Dose-related significant aberrations were breaks ($P < 0.01$), stickiness ($P < 0.05$) and autosomal univalents ($P < 0.05$). Figures 8-12 are representative photographs of significant aberrations.

Bone Marrow-Spermatocyte Aberration Correlations. Shown in Table 9 are the results of analysis of aberration correlation based solely on the treatment administered. No significant bone marrow-spermatocyte correlations were observed for control tissues. In contrast, cadmium-treated animals had significant positive

Table 7. Evaluation of specific chromosome aberrations after longer duration acute cadmium exposure of bone marrow cells.

Cadmium dose (mmoles/kg)	Time of sacrifice				
	Day 1	Day 3	Day 7	Day 30	Mean
	A. Proportion of aneuploids				
0.0252	0.266	0.237	0.280	0.796	0.395
0.0000	0.321	0.329	0.289	0.275	0.304
Mean***	0.294	0.283	0.284	0.540	0.349
S.E. = 0.012					
	B. Proportion of gaps				
0.0252	0.198	0.162	0.108	0.091	0.139
0.0000	0.126	0.103	0.112	0.095	0.109
Mean	0.162	0.133	0.110	0.093	0.124
S.E. = 0.010					
	C. Proportion of breaks				
0.0252	0.243	0.207	0.049	0.059	0.139**
0.0000	0.085	0.104	0.040	0.038	0.067**
Mean***	0.164	0.155	0.044	0.048	0.103
S.E. = 0.001					
	D. Proportion of rearrangements				
0.0252	0.003	0.031	0.069	0.042	0.036*
0.0000	0.007	0.010	0.018	0.018	0.013*
Mean**	0.005	0.021	0.044	0.030	0.246
S.E. = 0.001					
	E. Proportion of stickiness				
0.0252	0.018	0.013	0.065	0.059	0.038
0.0000	0.019	0.034	0.036	0.018	0.026
Mean	0.018	0.023	0.050	0.038	0.032
S.E. = 0.002					

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Table 7. (Continued)

Cadmium dose (mmoles/kg)	Time of sacrifice Day 1	Day 3	Day 7	Day 30	Mean
	F. Proportion of pulverizations				
0.0252	0.009	0.013	0.049	0.091	0.040*
0.0000	0.007	0.010	0.026	0.014	0.014*
Mean**	0.008	0.011	0.037	0.052	0.027
S.E. = 0.002					
	G. Proportion of despiralizations				
0.0252	0.055	0.017	0.073	0.042	0.046**
0.0000	0.028	0.018	0.018	0.023	0.022**
Mean	0.041	0.017	0.045	0.032	0.034
S.E. = 0.001					

*Significant at the 10% level.

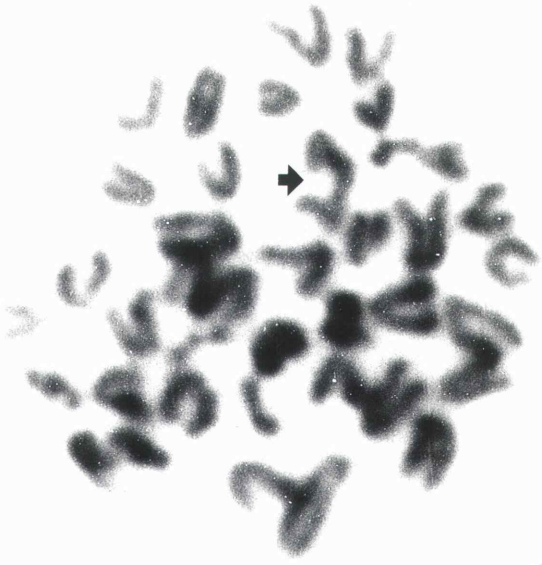
**Significant at the 5% level.

***Significant at the 1% level.

Figure 5. Mouse bone marrow mitotic figure with a dicentric (arrow). 2725X.

Figure 6. Mouse bone marrow mitotic figure that has become aneuploid. 2475X.

5



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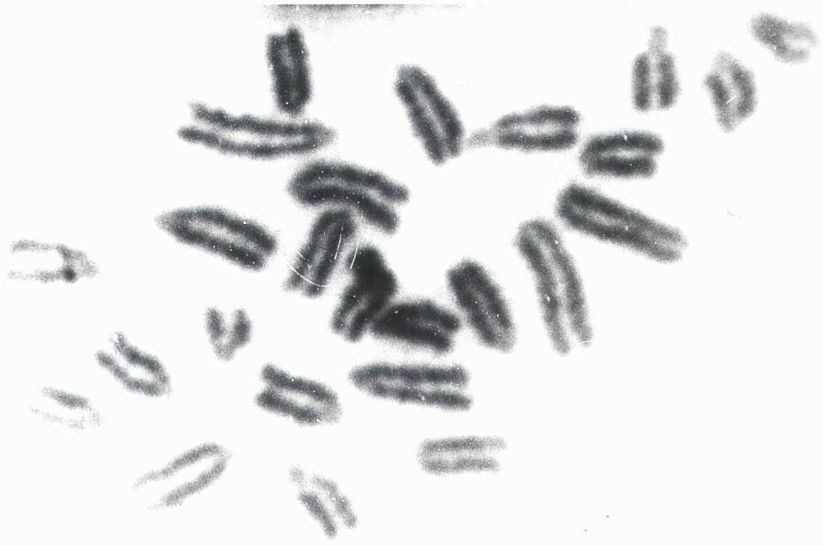

Table 8. Evaluation of specific chromosome aberrations after longer duration acute cadmium exposure of spermatocytes.

Cadmium dose (mmoles/kg)	Time of sacrifice Day 1	Day 3	Day 7	Day 30	Mean
	Proportion of aneuploids				
0.0252	0.059	0.045	0.032	0.044	0.045
0.0000	0.046	0.088	0.078	0.036	0.062
Mean	0.052	0.066	0.055	0.040	0.053
S.E. = 0.006					
	Proportion of gaps				
0.0252	0.105	0.061	0.009	0.009	0.046
0.0000	0.035	0.037	0.010	0.007	0.022
Mean*	0.070	0.049	0.009	0.008	0.034
S.E. = 0.002					
	Proportion of breaks				
0.0252	0.177	0.087	0.009	0.024	0.074***
0.0000	0.057	0.053	0.014	0.007	0.033***
Mean***	0.117	0.070	0.012	0.016	0.053
S.E. = 0.007					
	Proportion of rearrangements				
0.0252	0.031	0.056	0.004	0.004	0.024
0.0000	0.021	0.014	0.005	0.007	0.012
Mean***	0.026	0.035	0.004	0.006	0.017
S.E. = 0.001					
	Proportion of stickiness				
0.0252	0.086	0.016	0.006	0.004	0.028**
0.0000	0.010	0.004	0.005	0.007	0.006**
Mean**	0.048	0.009	0.006	0.003	0.017
S.E. = 0.0004					

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Table 8. (Continued)

Cadmium dose (mmoles/kg)	Time of sacrifice Day 1	Day 3	Day 7	Day 30	Mean
	Proportion of pulverization				
0.0252	0.017	0.006	0.009	0.013	0.011
0.0000	0.009	0.018	0.005	0.007	0.005
Mean	0.013	0.012	0.007	0.010	0.008
S.E. = 0.001					
	Proportion of XY univalents				
0.0252	0.051	0.079	0.070	0.087	0.072
0.0000	0.093	0.088	0.032	0.079	0.073
Mean	0.072	0.083	0.051	0.083	0.072
S.E. = 0.003					
	Proportion of autosomal univalents				
0.0252	0.032	0.060	0.060	0.038	0.119**
0.0000	0.029	0.021	0.047	0.011	0.027**
Mean	0.030	0.040	0.053	0.024	0.073
S.E. = 0.002					

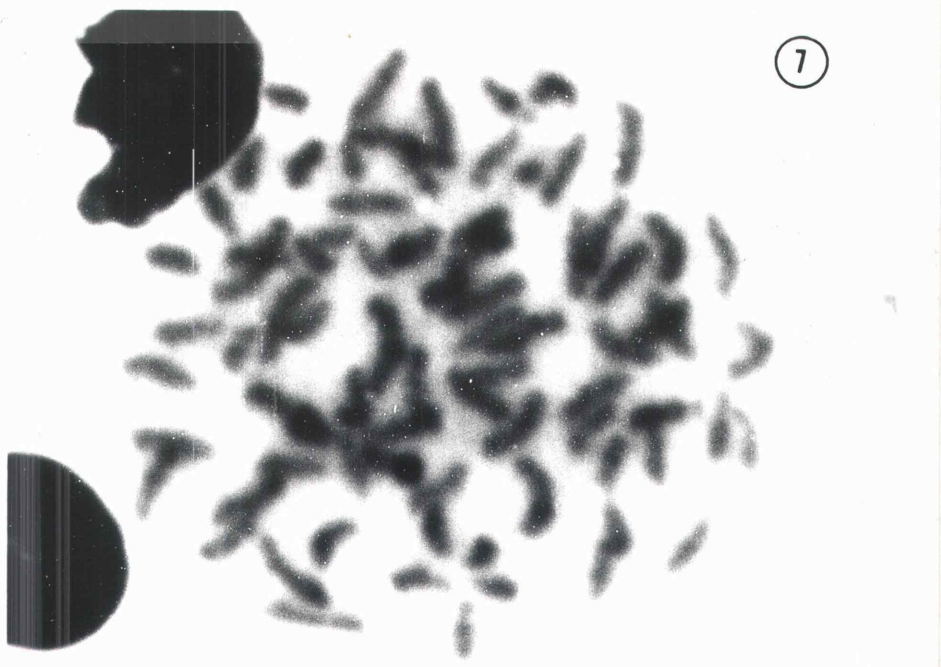
*Significant at the 10% level.

**Significant at the 5% level.

***Significant at the 1% level.

Figure 7. Mouse bone marrow mitotic figure that has become pulverized. 2725X.

Figure 8. Normal mouse spermatocyte meiotic figure. 2250X.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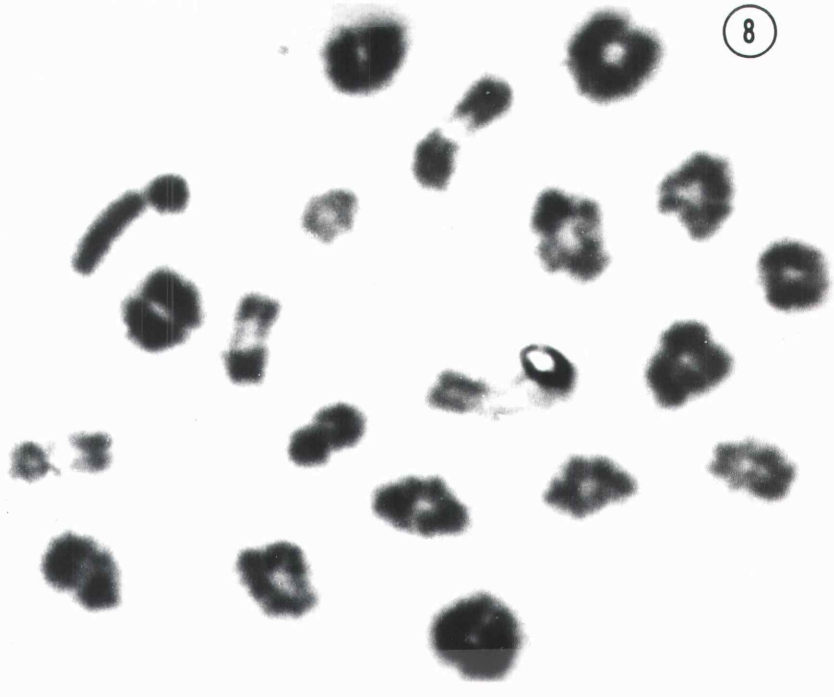
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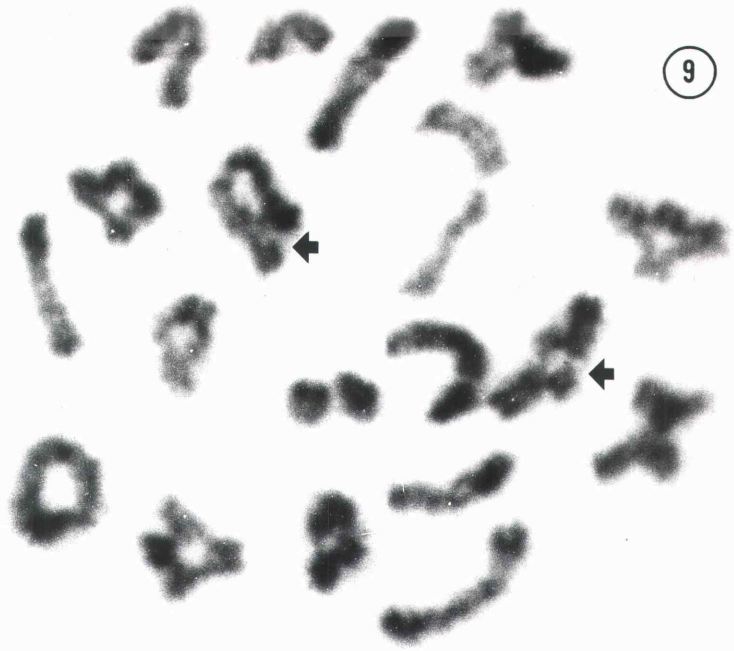
Figure 9. Mouse spermatocyte meiotic figure with gaps (arrows). 2025X.

Figure 10. Mouse spermatocyte meiotic figure with a break (arrows). An XY univalent is also present. 2475X.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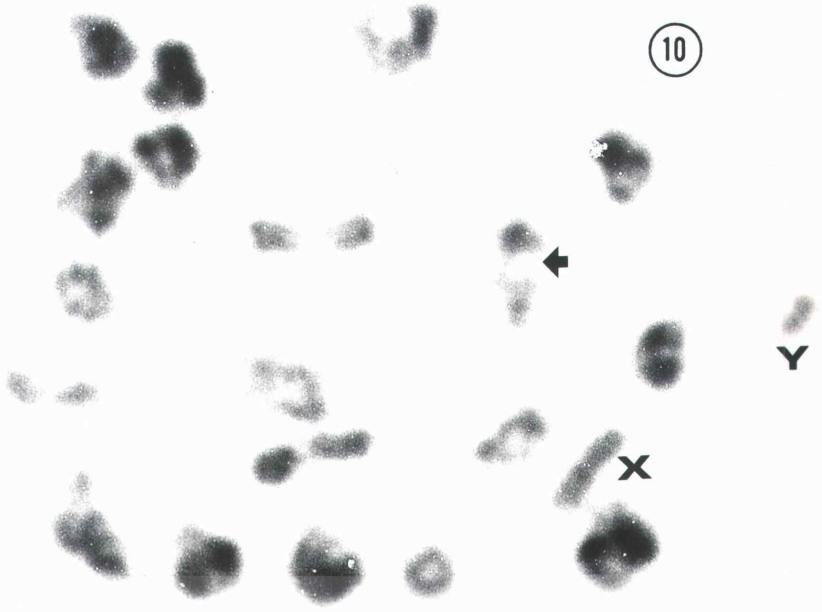
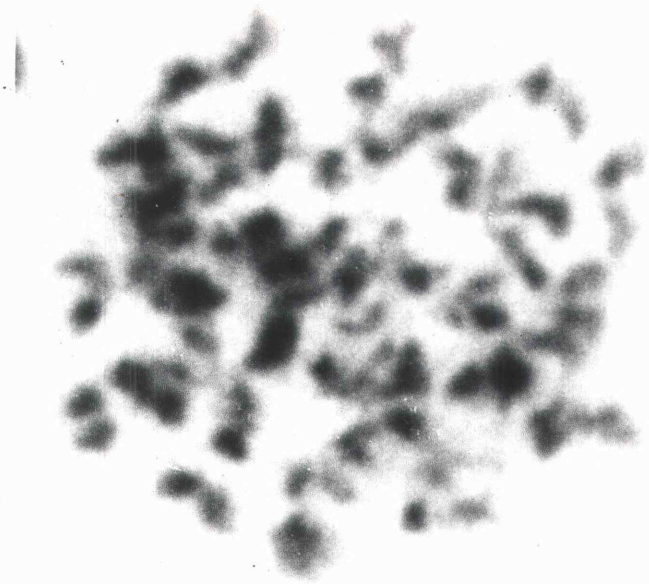
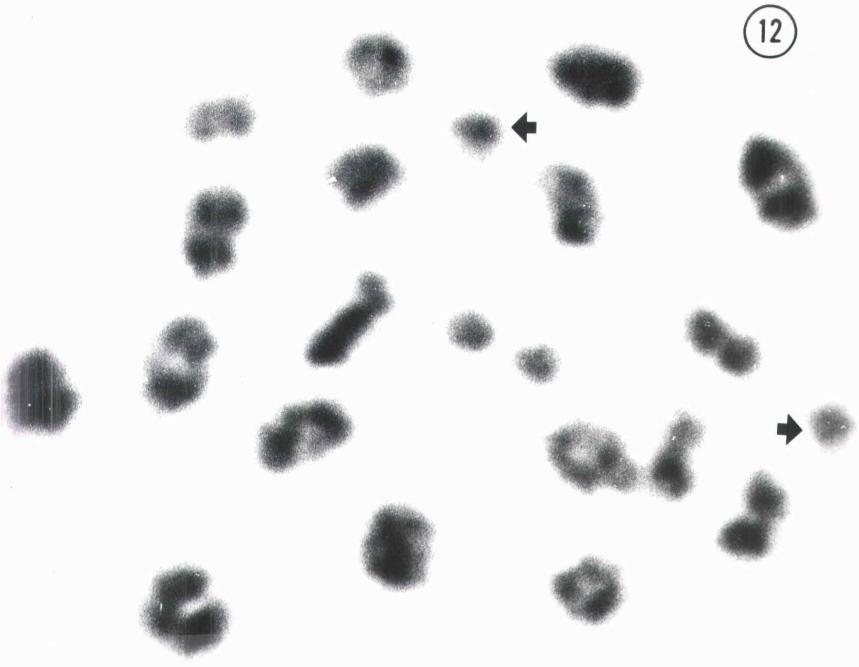

10
Y
X

Figure 11. Mouse spermatocyte meiotic figure with stickiness. 2725X.

Figure 12. Mouse spermatocyte meiotic figure with an autosomal univalent (arrows). 2725X.



(11)



(12)

Table 9. Results of spermatocyte-bone marrow correlation analysis with aberration frequencies grouped by treatment administered.

Cadmium dose (mmoles/kg)		Proportion of aberration[a]					
		ANG	G	B	R	S	P
0.0252	Correlation coefficient (r)	-0.17	0.39*	0.55**	-0.54**	-0.42**	-0.09
0.0000	Correlation coefficient (r)	-0.38	0.19	0.17	-0.34	0.18	-0.26

*Significant at the 10% level.

**Significant at the 5% level.

[a]ANG = aneuploid; G = gap; B = break; R = rearrangement; S = stickiness; P = pulverization.

correlations for gaps ($P < 0.1$, $r=0.39$) and breaks ($P < 0.05$, $r=0.55$) and significant negative correlation for rearrangements ($P < 0.05$, $r=-0.54$) and stickiness ($P < 0.05$, $r=-0.42$).

When the correlations of bone marrow and spermatocyte aberrations were carried out for each exposure time period, contrasting MTD treated animals with controls, significant correlations of specific aberrations were observed. In Table 10 it can be seen that no single aberration was significantly correlated with time, although bone marrow-spermatocyte comparisons showed good correlation of specific aberrations at given times.

When aberration frequencies were grouped by time and treatment, ignoring comparison of tissue from the same animal, the tests were sensitive to existing aberration differences between tissues without being affected by individual variability. This sensitivity was reflected in the number of significant correlations noted (see Table 11). A high frequency of significant differences among aneuploids (100%), gaps (62%), and stickiness (62%) was noted and no aberration class had a complete absence of significant differences. In all significant cases, except the significant difference between day one bone marrow and spermatocyte rearrangements, bone marrow cells had more aberrations than spermatocytes in their respective aberration classes.

The final statistical tests were done on individual tissue comparison and then grouped according to treatment and time. Table 12

Table 10. Results of spermatocyte-bone marrow correlation analysis with aberration frequencies grouped by time of exposure.

Time		Proportion of aberration[a]					
		ANG	G	B	R	S	P
Day 1	Correlation coefficient (r)	-0.15	0.16	0.32	0.26	-0.08	-0.34
Day 3	Correlation coefficient (r)	-0.38	0.58*	-0.04	-0.44	-0.35	-0.59*
Day 7	Correlation coefficient (r)	-0.08	0.30	-0.07	-0.28	0.31	-0.36
Day 30	Correlation coefficient (r)	-0.13	-0.35	-0.14	-0.61*	-0.70**	0.51

[a]ANG = aneuploid; G = gap; B = break; R = rearrangement; S = stickiness; P = pulverization.

*Significant at the 10% level.

**Significant at the 5% level.

Table 11. Results of spermatocyte-bone marrow comparison analysis with aberration frequencies from the tissues grouped by time and treatment.

Cadmium dose (mmoles/kg)	Tissue	Day 1	Day 3	Day 7	Day 30
		Proportion of Aneuploids			
0.0252	Testis	0.059	0.045	0.032	0.040
	Bone marrow	0.266***	0.237**	0.280**	0.792**
0.0000	Testis	0.046	0.088	0.065	0.037
	Bone marrow	0.321***	0.329**	0.289***	0.275**
		Proportion of gaps			
0.0252	Testis	0.105	0.061	0.009	0.004
	Bone marrow	0.198	0.161**	0.109**	0.091
0.0000	Testis	0.034	0.037	0.013	0.007
	Bone marrow	0.125*	0.103	0.112***	0.095***
		Proportion of breaks			
0.0252	Testis	0.176	0.088	0.009	0.023
	Bone marrow	0.243	0.207**	0.049**	0.048
0.0000	Testis	0.057	0.053	0.018	0.007
	Bone marrow	0.085	0.104	0.040	0.038*
		Proportion of rearrangements			
0.0252	Testis	0.031	0.056	0.004	0.004
	Bone marrow	0.003**	0.031	0.069**	0.042**
0.0000	Testis	0.021	0.014	0.005	0.007
	Bone marrow	0.007	0.010	0.018**	0.019
		Proportion of stickiness			
0.0252	Testis	0.022	0.016	0.006*	0.004
	Bone marrow	0.019	0.013	0.065***	0.059***
0.0000	Testis	0.010	0.004	0.005	0.007
	Bone marrow	0.019	0.034*	0.036**	0.018*

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Table 11. (Continued)

Cadmium dose (mmoles/kg)	Tissue	Day 1	Day 3	Day 7	Day 30
		Proportion of pulverization			
0.0252	Testis	0.018	0.005	0.009	0.023
	Bone marrow	0.009	0.013	0.049	0.091
0.0000	Testis	0.009	0.018	0.005	0.007
	Bone marrow	0.007	0.100	0.026*	0.014

*Significant at the 10% level.

**Significant at the 5% level.

***Significant at the 1% level.

Table 12. Results of spermatocyte-bone marrow correlation analysis with aberration frequencies from individual tissues grouped by time and treatment.

Time	Dose	Proportion of aberration[a]					
		ANG	G	B	R	S	P
Day 1	MTD[b]	-0.69	0.39	-0.03	0.57	-0.22	-0.88**
	C	0.73	-0.76	-0.63	0.29	0.62	0.39
Day 3	MTD	-0.49	0.42	-0.59	-0.95**	-0.51	-0.62
	C	-0.77	0.58	-0.31	-0.20	0.41	-0.93**
Day 7	MTD	-0.02	0.07	-0.17	0.22	0.08	-0.45
	C	-0.81	0.99**	0.82	-0.73	0.71	-0.69
Day 30	MTD	-0.85*	-0.32	-0.58	-0.09	0.09	0.32
	C	-0.90	-0.98**	-1.00	-0.53	-0.95*	0.98**

[a]ANG = aneuploid; G = gap; B = break; R = rearrangement; S = stickiness; P = pulverization.

[b]MTD = 0.0252 mmoles cadmium/kg body weight; C = 0.0000 mmoles cadmium/kg body weight.

*Significant at the 10% level.

**Significant at the 5% level.

summarizes these results. No significant occurrence of specific aberrations was noted over time or with different treatments. Of the isolated significant time and treatment correlations found in Table 12, gaps, stickiness and pulverizations predominated. The causes and significance of these aberrations, though not well documented, appear to relate to changes in chromosomal proteins as well as DNA (Goradia and Davis, 1977; Klasterska, Natarajan and Ramel, 1976).

Organ Weight-Body Weight Ratios

Results of the analysis of organ weight-body weight ratios from the acute exposure experiment are summarized in Table 13. Spleen mean ratios of weights at 6 and 24 hours were significantly different ($P < 0.05$ and $P < 0.01$) due to dose effects. Liver mean ratio of weights at 6 hours were significantly higher ($P < 0.01$) and at 24 hours also different ($P < 0.01$) when examined for changes due to dose effects. Subacute exposure results (see Table 14) were not significantly different due to dose effects.

Table 15 summarizes the organ weight-body weight analysis for the exposure duration experiment. Liver mean weight ratios increased significantly ($P < 0.01$) over time. The spleen ratios were also significantly different ($P < 0.10$), but it was day three spleens which were significantly larger than the other three time periods. No significant changes were noted for kidney or testis.

Table 13. Organ weight/body weight ratio results from the acute exposure study.[a]

Cadmium dose (mmoles/kg)	Time of sacrifice 6 hours	24 hours	48 hours	Mean
		Liver		
0.0252	54.98***	51.37***	50.92	52.42
0.0126	53.95***	54.79***	54.54	54.42
0.0055	52.96***	52.37***	53.49	52.84
0.0000	49.88***	52.75***	51.08	51.24
Mean	52.94***	52.82***	52.50	52.75
S.E. = 1.002				
		Kidney		
0.0252	5.95	6.15	6.01	6.03
0.0126	6.22	6.14	6.41	6.25
0.0055	4.87	6.25	6.61	5.91
0.0000	6.02	6.29	6.21	6.17
Mean	5.76	6.21	6.31	6.09
S.E. = 0.096				
		Spleen		
0.0252	2.11**	2.70***	3.24	2.68
0.0126	2.23**	2.42***	2.74	2.46
0.0055	2.45**	2.47***	3.61	2.84
0.0000	2.29**	2.86***	2.88	2.67
Mean	2.27	2.61	3.11	2.66
S.E. = 0.145				
		Testis		
0.0252	3.95	3.79	4.16	3.96
0.0126	4.05	4.17	4.04	4.08
0.0055	4.07	4.14	4.39	4.20
0.0000	4.31	4.12	4.10	4.17
Mean	4.09	4.05	4.17	4.10
S.E. = 0.116				

[a] All values times 10^{-3}.

** Significant at the 5% level.

*** Significant at the 1% level.

Table 14. Organ weight/body weight ratio results from the subacute exposure study.[a]

Organ	Cadmium dose (mmoles/kg)				Mean	S.E.
	0.0252	0.0126	0.0055	0.0000		
Liver	53.79	49.18	51.03	46.95	50.23	2.939
Kidney	6.14	6.37	6.44	5.84	6.30	0.204
Spleen	3.26	2.84	2.81	2.19	2.77	0.407
Testis	3.99	4.34	4.26	4.01	4.15	0.147

[a]All values times 10^{-3}.

Table 15. Organ weight/body weight ratio results from the exposure duration study.[a]

Organ	Dose	Day 1	Day 3	Day 7	Day 30	Mean
Liver	MTD*	44.89	49.44	53.22	52.92	50.18
	C	45.49	44.54	50.30	53.57	48.47
	Mean***	45.19	46.99	50.97	53.58	49.32
	S.E. = 1.673					
Kidney	MTD	6.18	6.60	6.90	6.97	6.66
	C	6.56	6.61	6.53	7.26	6.74
	Mean	6.37	6.61	6.64	7.10	6.70
	S.E. = 0.1748					
Spleen	MTD	2.57	3.33	2.39	2.61	2.72
	C	3.05	2.88	2.42	2.40	2.68
	Mean*	2.81	3.11	2.40	2.58	2.70
	S.E. = 0.1741					
Testis	MTD	3.21	3.65	3.78	3.81	3.61
	C	3.59	3.63	3.46	3.54	3.55
	Mean	3.40	3.64	3.68	3.71	3.58
	S.E. = 0.1158					

[a]All values times 10^{-3}.

*Significant at the 10% level.

***Significant at the 1% level.

Histology

No histological changes were observed in the testis or kidney in MTD animals at days 1, 3, 7 and 30 exposures. As early as day 1 the livers of treated animals showed vascular congestion, necrosis, heteropycnotic nuclei, and had begun fatty infiltration. In addition, by day 7 livers from two animals had bile duct hyperplasia. On day 30 the livers were still necrotic and bile duct hyperplasia was noted in approximately half of the animals. Spleens were histologically normal on day 1. By day 3 proliferation of the white pulp was noticeable and persisted through day 30.

Blood Cell and Bone Marrow Counts

When differential blood counts from the pilot study were compared at days 1, 3, 7 and 30, no changes in white cell populations were noted. Considerable numbers of Howell-Jolly bodies in individual treatment and control animals and the presence of polychromasia in treatment animal red cells suggested possible erythropoietic stress.

Total bone marrow cell counts and reticulocyte counts from MTD treated animals were significantly ($P < 0.05$) greater than controls 30 days after exposure (see Tables 16 and 17). This information, when considered with the blood cell counts, suggested that the

cell proliferation observed was occurring in the erythropoietic stem cells.

Table 16. Bone marrow total cell counts[a] after 30-day exposure to cadmium chloride (0.0252 mmoles/kg).

	$\bar{x}$	S.D.	Number of animals
Cadmium chloride	15.78*	3.92	10
Control	11.61	2.43	10

[a]Total cells counted (x 10^3) in 20 lambda bone marrow sample.

*Significant at the 5% level.

Table 17. Reticulocyte counts after 30-day exposure to cadmium chloride (0.0252 mmoles/kg).

	$\bar{x}$	S.D.	Number of animals
Cadmium chloride	7.95	1.40	10
Control	0.98	0.33	10

*Significant at the 5% level.

IV. DISCUSSION

Dosage Determination

The cadmium chloride dose, 0.0252 mmoles or 2.8 mg cadmium/kg body weight, tolerated by the C57Bl/6J males was considerably less than has been reported for other testicular necrosis-sensitive and resistant strains. Gunn and Gould (1970) and Christensen (1974) reported doses of 5-6 mg/kg tolerated by most strains.

Changes in appearance and behavior noted at highest cadmium concentrations (0.101, 0.0505, 0.0252 mmole cadmium/kg) were similar to those recently recorded by Der, Fahim, Yousef and Fahim (1976, 1977) in chronic exposure studies with rats.

The gross pathological changes of the liver found in the autopsied animals, as discussed later, correspond with microscopic observations of Colucci, Winge and Krasno (1975) and of Hoffman, Cook, DeLuzio and Coover (1975), which revealed focal necrosis with hetero-pycnotic staining of nuclei and fatty infiltration. Lucis and Lucis (1969) reported that within 24 hours approximately 90% of ^{109}cadmium chloride dose administered to male C57B/6J mice localized in liver, kidney, and pancreas, with approximately 50% of the dose being localized in liver. Because the major portion of the total body burden of cadmium was found in the liver, and the liver is an organ of

detoxification, it would be expected that it could be among the organs most seriously affected by high cadmium doses.

The discolored, pale, yellowed kidneys of the autopsied animals were similar to those described by Itokawa, Abe, Tabei and Tanaka (1974) who reported relative increase in the kidney weight of treated animals associated with degeneration of glomeruli and tubular epithelium. Because the tissues of this study were obtained from dead animals, reliable histological evaluation was not possible. Kidneys examined in both acute exposure and exposure duration studies with lower cadmium doses showed neither gross pathological nor microscopic changes.

Kidney cell alterations have been found more consistently in cases of chronic toxicity (Cousins, Squibb, Feldman, de Bair and Silbon, 1977). The pathological effects of cadmium in kidney may be due to metallothionein-bound cadmium rather than free cadmium. Tanaka, Sueda, Onosaka and Okahara (1975) reported that free cadmium mainly accumulated in the liver while bound cadmium was accumulated in the kidney. Nordberg, Goyer and Nordberg (1975) showed that injected metallothionein-bound cadmium caused severe tubular changes, while animals receiving free cadmium had no pathological change.

Splenic enlargement may have been due to an immunological response, to a generalized hyperplasia, or to hyperemia. Histology

was not possible on these specimens, but histologic examination done for the lower doses given in the acute and exposure duration experiments indicated proliferation of cells in the white pulp. This proliferation would suggest an immunological response. The reports of Koller, Exon and Roan (1975, 1976) on the effects of cadmium on antibody synthesis by the spleen of Swiss Webster mice, confirmed the presence of an immunological response after acute cadmium exposure. Their studies revealed that a single acute exposure stimulated IgM antibody production whereas chronic long-term exposure had an immunosuppressive effect.

Mutagenic Testing

In the initial analysis of grouped data the increase in structural aberrations occurring with time in the acute exposure, subacute exposure, and exposure duration experiments could be anticipated after exposure to a potential chemical mutagen such as cadmium for the following reasons: there was sequestering of cadmium in the bone marrow (Lucis and Lucis, 1969), there is no homeostatic mechanism to control final tissue concentrations of cadmium (Friberg et al., 1974), and the biological half-time of cadmium is long, about 100 days for mice (Friberg, 1974). In no other study has a time-related mutagenic effect been examined. In the studies of Deknudt and Leonard (1975), the time of exposure was recorded for each human but was not considered in the evaluation of aberration frequencies.

No significant dose-related aberrations were found in the initial grouped analysis. This may indicate that cadmium really had no mutagenic effect, that cadmium indirectly acted as a mutagen or would require synergistic factors to be mutagenic, or that a threshold response with considerable variability had occurred. However, for statistical analysis time groups were pooled for each dose, and because of this significant dose-related events may have been overlooked.

Analysis of the total number of aneuploids per total number of cells examined was not statistically significant. Evaluation of total aneuploids as a mutagenic manifestation was not a clearly defined procedure and could again have masked severely aberrant classes of cells. By strict definition any mouse bone marrow cell would be aneuploid if it had some chromosome count other than 40, and the analysis of this study was based on this figure. From previous studies (Doyle et al., 1973), it is known that a model distribution of chromosome counts would be found, even in control animals.

The lack of significant differences between the total number of abnormal cells found per total cells examined was also probably due to grouping of unrelated abnormalities in this comparison.

Examination of specific aberration data revealed significant increases in breaks with time and dose. These results corresponded with the findings of Shiraishi and Yosida (1972) with Itai Itai patient

lymphocyte chromosomes; Bauchinger et al. (1976) with smelting plant worker lymphocytes; and Shiraishi, Kurahashi and Yosida (1972) with in vitro cadmium-treated human lymphocytes. It should be noted that the number of rearrangements may have been underestimated because the acrocentric mouse chromosomes were almost identical in size and, as such, chromatid exchanges would not be detectable without the use of chromosome banding procedures (Leonard, 1973).

Subacute exposure also resulted in dose-related occurrences of breaks. This was strongly suggestive of fairly consistent mutagenic action of cadmium. Deletion means were significantly different, but not in a dose-effect related way. The intermediate dose group had fewer deletions, the normal exposure dose group had more deletions and the maximum tolerated dose group was not different from the control group. One can only speculate as to how this effect could come about. The most likely explanation, based on the interrelatedness of aberrations resulting from the breaking of the chromosome strand, would be a shifting of the occurrence of deletions in favor of some other form of chromosome breakage. Vig (1975) reported that aberration positions may be affected by nuclear membrane attachment during cell cycle phases, with membrane-attached regions being more susceptible to breakage and that more variable radiomimetic actions would be found during the S phase. Vig's findings may also apply to

findings in this study. As will be discussed in a following section, total bone marrow counts revealed a cadmium-related cell proliferation which might shorten the G_1 phase of the cell cycle.

In assessing the mutagenic properties of various agents, two factors are of prime consideration (Cohen and Hirschhorn, 1971). The first factor is whether or not the agent induces damage and secondly whether the agent can lead to stable aberrations. The structural rearrangements enumerated in this cadmium study included both unstable abnormalities, such as chromatid breaks and pulverization, and stable aberrations, such as translocation. Observed breaks and deletions indicate that cadmium definitely induces damage. Possible explanations for this structural damage are that cadmium acts directly to damage DNA and/or protein components of the chromosome or that cadmium inhibits repair enzyme systems.

Several studies indicate cadmium may act directly with DNA. The studies of Mizuhira and Kimura (1975) indicate cadmium can be detected in heterochromatic portions of liver cells 2 hours after intraperitoneal injection of cadmium chloride. Sissoeff et al. (1975) detected preferential localization of cadmium in repetitive DNA sequence of tobacco crown gall cells. Finally Stoll, White, Miya and Bosquet (1976) determined that cadmium directly altered DNA melting profiles of calf-thymus DNA. Their interpretation of the

profiles was that a more complete unwinding of DNA occurred in the presence of cadmium.

The question of whether cadmium leads to stable aberrations has not been conclusively answered by previous in vitro studies of somatic tissues, by the studies of Gilliavod and Leonard (1975) or Shimada et al. (1976) with germinal tissues or in the study reported now in which in vivo exposure of both somatic and germinal tissues was examined. Two factors may influence the detection of stable rearrangements. First reciprocal exchanges in mouse cells are not readily detectable without the use of chromosome banding procedures (Leonard, 1973). Secondly since only very low frequencies of stable rearrangements (<0.5-2%) may be detected, it would be desirable to use larger samples to detect adequate quantities of the rarer occurring events (B. McClanahan, personal communication). As Kihlman (1971) noted both stable translocation figures and unstable dicentrics could be observed in metaphase configurations; both were observed in very low combined frequency of 0.3 to 6.9%. The relatively high frequency of breaks with no significant observation of stable rearrangements is not uncommon (Cohen and Hirschhorn, 1971). The failure to form stable rearrangements when other radiomimetic effects are produced could be related to repair enzyme inhibition or to unfavorable ionic changes in the nucleus which prevent strand healing. It is commonly recognized (Legator and Zimmering, 1975) that

". . . even those chemicals that produce or potentiate chromosome breakage without ensuring rearrangements could constitute a potential risk for future generations" and should be reported.

With the ultrastructural studies of Brinkley and Hittelman (1975) indicating that not all breaks scored according to conventional standards of misalignment and a discontinuous span the width of the chromosomes, are completely discontinuous, some question may arise the actual observation of unstable aberrations. However in the same study chromosomes scored as gaps according to the same conventions were found to be distinct discontinuous breaks. Light microscopic examination alone will not be sufficient to be absolutely certain that breaks rather than attenuated gaps have occurred. Cohen and Hirschhorn (1971) note that no healing of extremely attenuated gaps has been noted. Tolbey and Hecht (1968) recommend both direct microscopic and photomicroscopic scoring of aberrations to most accurately determine aberration at light microscopic resolution. This method was used in this study for doubtful cases. Further study of the nature of achromatic gaps is necessary, as well as ultrastructural examination with scanning and transmission procedures, before any study can report the occurrence of breaks accurately.

Exposure Duration Experiment

Bone Marrow Analysis

When bone marrow cells were exposed to cadmium for longer periods, time-related increases in the number of breaks, rearrangements and pulverizations occurred. Dose-related increases were noted in these three classes also as well as an increase in the number of despiralized plates. It is noteworthy that with increasing exposure time the occurrence of physiological effects, i.e., stickiness and despiralization, appeared. That these aberrations may have come about by prolonged colcemid or hypotonic treatment has been considered. Bosman, Van de Ploeg, Schaberg and Van Duijn (1975) found no colcemid-related stickiness or contraction of chromosomes in a study designed to determine the effects of prolonged colcemid treatment on human lymphocyte chromosomes. Despiralization, however, can be caused by prolonged hypotonic treatment (Goradia and Davis, 1977). Bone marrow processing, particularly colecmid treatments, was carried out with great care taken to avoid hypotonic despiralization. The appearance and paucity of despiralized plates per slide seemed to indicate that some other agent, possibly the cadmium, was causing the despiralization. Control slides confirm this and the findings are consistent with the results of Rohr and Bauchinger (1976). Also supporting the belief that despiralization is a cadmium-induced

effect is the cadmium directed observed increase in DNA unwinding of calf-thymus DNA examined for DNA melting profiles (Stoll et al., 1976).

The significant increase of chromatid breaks through day 30 of the experiment would not be expected with nonpersistent chemicals. However, with cadmium, both selection against aberrant cells and persistent mutagenic effects on new daughter cells and previously exposed cells, would be continuing.

Spermatocyte Analysis

Gaps, breaks, rearrangements, and stickiness increased with time in the spermatocytes. A dose-related increase in breaks, stickiness and autosomal univalents was also noted. These aberration occurrences were the first noted for spermatocytes exposed to cadmium. In the only other study done with cadmium-exposed spermatocytes, Gilliavod and Leonard (1975) scored only translocation figures and found absolutely no figures. Gilliavod and Leonard noted in their discussion that it was rather surprising that no genetic effects were produced since the cadmium was shown to kill spermatogonia and to induce testicular interstitial cell tumors after acute exposure.

The possible transmission of these abnormalities was not ascertained in this study. There has been some question as to the transmission of heritable change due to gaps (Brinkley and Hittelman,

1975). Rearrangements, stickiness, and univalents produce aneuploid gametes or may prevent the continuation of cell division. Determination of the transmission potential of univalents may be particularly difficult because in addition to failure to synapse a variety of circumstances, such as chromosome number in the species and the makeup of individual chromosomes, can alter the events of the first meiotic division (Polani and Jagiello, 1976).

Bone Marrow-Spermatocyte Correlation

Several statistical approaches were used in correlating bone marrow and spermatocyte aberrations of the exposure duration experiment. In the first approach, data were grouped according to treatment alone, i.e., MTD or control. No correlation existed between the bone marrow and spermatocyte aberrations found in control animals. This was to be expected if the aberrations were random spontaneous occurrences. The cadmium-treated animals had significant positive correlation for gaps and breaks and negative correlations for rearrangements and stickiness. It appeared that the rearrangements, because of their rare occurrence and the subjective identification required (Leonard, 1973), would be difficult to find in spermatocytes even when rearrangements were increasing in the bone marrow cells. This finding might help to explain the negative observations of Gilliavod and Leonard (1975).

The second approach to correlating bone marrow-spermatocyte data was to group according to the exposure time period. No consistent correlation was found for any aberration for the four exposure periods. The resolution of this test was limited because of the grouping of treatments at each time period.

A third approach used to relate the aberration frequencies of the two tissues was to group the data by time and treatment without regard for relating the two tissues of individual animals. The data correlations were nearly unanimous in indicating that the spermatocytes will have significantly fewer aberrations than bone marrow cells with time and dose constant. A hesitant interpretation should be made beyond this because, although statistically significant, these correlations say very little about biologically important events. The total number of aberrations being compared is extremely small in some cases, e.g., rearrangements. The correlation of two very minor aberration totals would not mean a major effect, e.g., increased teratogenic events or mutant offspring.

The final statistical test done related the individual tissues before grouping by treatment and time for analysis. No significant relationship could be demonstrated indicating that there was no correlation of cadmium-induced aberration between the organs within an animal. Extrapolation of information on bone marrow to spermatocytes should not be done.

Discussion of Results as Related to Zinc Deficiency

Because of the chemical antagonism between zinc and cadmium one may suspect zinc deficiency to be responsible for observed chromosomal damage rather than direct cadmium action. General symptoms of zinc deficiency, anorexia, poor growth, alopesia (Fallin, 1958; Soloman, Rosenfield, Jacob and Sandstead, 1976) testicular atrophy and spermatogenic arrest (Fisher, 1975) were not observed. Among the chemical derangements associated with zinc deficiency growth retardation are severe depression of RNA and protein synthesis, DNA doubling, accumulation of unusual peptides, amino acids, nucleotide and protein (Falchuck, Fawcett, and Vallee, 1975). Chesters (1974) reported that in zinc deficiency DNA synthesis is impaired. Although no specific information is available on zinc-cadmium antagonism in bone marrow, the cell proliferation of erythroblasts and the maintenance of white blood cell population observed in this study would suggest that bone marrow cells were not experiencing zinc deficiency. Studies do indicate that zinc increases in the testis after cadmium injection (Webb cited by Bremner, 1974) and that cadmium has no effect on thymidine incorporation by spermatogenes (Dixon, Lee and Sherins, 1976). Neither testicular atrophy nor spermatogenic arrest was noted with our treatments.

Evaluation of the Mutagenic Strength of Cadmium

In discussing the results of the present *in vivo* cytogenetic testing, it must be stated that the mutagenic strength of any agent is not based on a single method of detection of genetic damage but instead on a battery of detection methods (Legator and Zimmering, 1975). The major procedures to be used are *in vitro* testing with phage, bacteria and yeast, *in vitro* testing of microsomal activation, host-mediated assays, *Drosophila* testing for sex-linked recession, translocation, nondisjunction and chromosomal loss, and studies in intact animals such as repair, cytogenetic, dominant lethal and specific locus tests. To date cadmium has been tested *in vitro* with *Bacillus subtilis* (Nishioka, 1975); in *Drosophila* for sex-linked recession and chromosome loss (Ramel and Friberg, cited by Friberg et al., 1974) and in intact animals for changes in repair mechanism (Ramel and Friberg, cited by Friberg et al., 1974), for dominant lethal (Epstein et al., 1972; Gilliavod and Leonard, 1975; Suter, 1975) and cytogenetic effects (von Rosen, 1953; Oehlkers, 1953; Shiraishi et al., 1972; Paton and Allison, 1972; Shiraishi and Yosida, 1972; Doyle et al., 1973; Bui et al., 1975; Deknudt and Leonard, 1975; Bauchinger et al., 1976; Rohr and Bauchinger, 1976; Gilliavod and Leonard, 1976; Shimada et al., 1976).

In the in vitro testing of *Bacillus subtilis*, cadmium chloride but not cadmium nitrate was found to damage DNA. No sex-linked

recessives, chromosome loss or repair mechanism changes were observed in Drosophila fed cadmium chloride. The dominant lethal tests of Epstein et al. (1972) and Suter (1975)were definitely not significant with cadmium chloride. Gilliavod and Leonard (1975) also reported insignificant dominant lethal results with cadmium chloride when testing at the 5% level of significance but their results were significant at the 6% level. Because of Gilliavod and Leonard's results, further dominant lethal testing with larger samples and longer times of exposure would be warranted. Previous in vivo cytogenic studies neither confirm nor deny the mutagenic potential of cadmium. The in vivo studies of this report are suggestive of the occurrence of significant chromosomal damage.

Considering the recommended battery of procedures to be tested and these results reported for cadmium, further testing is necessary to determine the actual mutagenic strength .

Toxicological Monitoring

Organ Weight-Body Weight Ratios Analysis

Toxicological effects of cadmium were monitored in three ways besides the mutagenic testing, since it was considered most important to be certain that the reported cadmium damage had taken place in those animals being examined for aberrations. The first of these monitoring procedures was the analysis of organ weight-body weight

ratios. Animals exposed to toxic compounds might be expected to show changes in organ weights. If body weights were not appreciably altered, as is true in this study, organ weight-body weights ratios are dependable expressions for reducing variability of the data and for removing bias due to different body weights (Stevens, 1976). The mean spleen ratios at 6 and 24 hours were significantly lower when compared for changes due to dose at set time periods after acute exposure.

When time-related changes were analyzed, no significant difference was found. There appeared to be great variability in response in the earlier two time periods which may be a manifestation of a greater difference in individual body responses to the initial cadmium exposure.

At 6 hours the liver weight ratio means differed significantly and this weight difference was a dose-effect response. The livers at 24 hours were also significantly different in size with the ITD level having the largest mean and the MTD having the smallest. It appeared, however, that vascular congestion of the liver may have begun by 6 hours after treatment in the MTD animals and that a slower but progressive congestion occurred in the ITD animal livers at 24 hours. Degeneration of MTD livers at 24 hours and possibly the detection of congestion in the ND animals at 48 hours would account for the shift in mean weight ratios. Histological examination of the

livers supported this explanation. When weights were grouped by dose, irrespective of length of cadmium exposure, no significant differences among treatment groups were observed. This may be due to the fact that once a threshold dose was received by the liver, the rate of degeneration could not be increased.

With the split-dose subacute exposure, no significant dose effect organ weight differences were noted. It has been shown that small doses of cadmium can trigger the production of metallothionein, particularly by the liver, as a means of binding cadmium and decreasing the damage incurred by subsequent exposure (Friberg et al., 1974; Sabbioni and Marafante, 1975).

In the exposure duration experiment, liver ratio means increased significantly over time. This may only reflect continued growth of the liver. From other studies, particularly Der, Fahim, Yousef and Fahim (1976, 1977), it seemed likely that the differences were not cadmium-related. Spleen ratios were also significantly different. In this case, day 3 spleen ratios were greater than the other three periods. Histological examination confirmed a marked proliferation of the white pulp at day 3. Koller, Exon and Roan (1976) reported that with acute cadmium chloride exposure, IgM synthesis by the spleens of Swiss Webster mice was increased beginning at day 3. The organ weight-body weight ratios and histological findings of this study both confirmed a specific spleen response on day 3 and

the histological findings lent support to the findings of Koller, Exon and Roan.

No significant organ weight changes were noted in the kidney or testis. Kidney damage will normally be the result of a longer exposure to cadmium (Friberg et al., 1974). Nordberg, Goyer and Nordberg (1975) reported that it was cadmium-metallothionein complexes that caused kidney damage. The exposure duration of the studies reported here would not be sufficient for detectable histological damage to occur. The testes of C57B1/6J mice had previously been reported (Gunn and Gould, 1968) to be resistant to cadmium-induced vascular necrosis found in other mammals.

Histology

Histology was a second monitoring procedure used in this study. As noted already, no histological changes were observed up to day 30 in the testis or kidney in animals receiving 0.0252 mmoles cadmium/kg body weight. However, both liver and spleen were severely affected by this dose. As early as day 1, livers had necrotic areas, heteropycnotic nuclei, fatty infiltration and vascular congestion. These observations had been made previously by Colucci et al. (1975) and Hoffman et al. (1975), and were good indications of acute cadmium toxicity. By day 30 the occurrence of bile duct hyperplasia had begun to occur. The significance of this observation is unclear.

Spleen histology has been briefly mentioned in the previous section. The most important feature was the proliferation of the white pulp that involved both an increase in nodes and an increase in node size. This proliferation was noted on day 3 and persisted through day 30.

Blood Cell and Bone Marrow Counts

The final monitoring procedures were done on blood and marrow cells to determine if a similar population of cells was being acted upon at all exposure times.

From the differential blood cell counts, it was observed that white cell populations were not significantly effected by an *in vivo* cadmium exposure. Howell-Jolly bodies, nuclear inclusions found in red blood cells, occurred in extremely variable numbers in treatment animals and also occurred in two control animals. Although in other mammals these inclusions have been good indicators of bone marrow stress, in mice they have been commonly found in healthy animals and cannot be taken as evidence of stress in the erythropoietic system (Schermer, 1967). Polychromasia was also pronounced in the animals examined. Schermer (1967) stated that in young mice this may normally occur. It was only with the total bone marrow counts taken at day 30 that it was definitely ascertained that bone marrow stress, manifested as an increased proliferation, occurred in cadmium chloride-treated animals. From the observations here and the work of Johns, Sanders and Powers (1977), it is clear that red cell lysis does

occur after cadmium exposure. A dose-effect response was noted by Johns, Sanders and Powers (1977). In the blood cadmium has been known to concentrate on circulating red cells (Friberg et al., 1974). These results may indicate that with prolonged exposure red cell precursor population may predominate the bone marrow cells being examined for chromosomal changes. If differences in sensitivity exist between red and white blood cells these may be reflected in this study. At present it is not possible to distinguish mitotic white cells from the mitotic red cells of the bone marrow (H. Ragan, personal communication) to determine if, in fact, metaphases of different populations were examined.

Time Course of Mutagenic and Histologic Events

The onset of genetic damage in bone marrow cells was 6 hours and 24 hours in spermatocytes. Detectable light microscopic changes in the liver first occurred at 24 hours while changes in the spleen were noted only after 72 hours. This time course indicates that the genetic damage is not dependent on the damage of other organs and may suggest that the genetic events are primary rather than secondary. The occurrence of genetic damage as early as 6 hours would require that cadmium reach target cell nuclei in that time. Mizahira and Kimura (1973) noted that cadmium can be detected in the heterochromatin of liver cells 6 hours after injection. Shaikh and Lucis (1972) found that cadmium influx into testes of mice ceased within 6 hours after

subcutaneous injection but remained fairly constant until termination of the experiment at 25 days.

V. SUMMARY

The objectives of this study were to determine if cadmium would induce chromosomal aberration, to determine if simultaneous aberration events occurred in somatic and germinal tissue, and to determine an estimated minimum exposure time required for significant chromosomal change.

Bone marrow chromosome aberrations, specifically breaks and deletions, were found to increase after acute cadmium exposure both at MTD and normal exposure levels. Subacute exposure also resulted in increased occurrences of breaks, deletions, and despiralization.

With longer in vivo exposure to cadmium bone marrow cells continued to show increased numbers of breaks, as well as a physiological effect, despiralization, and more severe break-related aberrations; rearrangements and pulverization. In spermatocytes of the same animals, gaps, breaks, rearrangements, stickiness, and autosomal univalents were the principle aberrations.

Correlation of bone marrow and spermatocyte aberrations indicated that in treated mice significant relationships existed for gaps, breaks, rearrangements and stickiness in the tissues. No significant correlation existed between the tissues over time. When the correlation was examined between tissues at the same time and treatment a number of correlations were found. Individual animal comparison showed no significant aberration correlation.

An estimate of the minimum exposure time required to produce chromosomal damage, based on the acute exposure experiment, would be 6 hours for bone marrow. This was confirmed by the exposure duration experiment. Spermatocytes also had chromosomal damage within 24 hours.

Procedures used to determine the occurrence of cadmium damage were evaluation of organ weight-body weight ratios, histological examination of target organs and blood cells, and bone marrow counts. All three procedures indicated toxic effects had occurred in acute, subacute and exposure duration experiments.

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APPENDICES

APPENDIX I

National Institute for Occupational Safety and Health Definition of a Toxic Chemical (Christensen, 1974)

A chemical is defined as toxic if it

demonstrates the potential to induce cancer, tumors, or neoplastic effects in man or experimental animals; to induce a permanent transmissible change in the characteristics of an offspring from those of its human or experimental animal parents; to cause the production of physical defects in the developing human or experimental animal embryo; to produce irritation; to diminish mental alertness or motivation; or to endanger the life of man when he is exposed to the substance via the respiratory tract, skin, eye, mouth or other routes in any quantity reported. . . .

APPENDIX II

Mutagenic Testing Protocol Used for the Study of Cadmium Chloride Effects in the Bone Marrow of C57B1/6J Male Mice (modified after Nichols, Moorhead, and Brewen, 1972).

Treatment	Number of animals to be used Time killed after $CdCl_2$ administration (hr)		
(mmole Cd/kg body wt)	6	24	48
A. Acute Study[a]			
0.0252[b]	5	5	5
0.0126[c]	5	5	5
0.0055[d]	5	5	5
deionized distilled water[e]	5	5	5
B. Subacute Study[a]			
	5 injections, 24 hr apart. Animals sacrificed 6 hr after last injection.		
0.0252		5	
0.0126		5	
0.0055		5	
deionized distilled water		5	
C. Exposure Duration[f]			
	Time killed after $CdCl_2$ administration		
	Day 1	Day 3	Day 7	Day 30
0.0252	4	4	4	4
deionized distilled water	4	4	4	4

[a] 50 Metaphase plates scored per animal

[b] Maximum dose tolerated

[c] Intermediate level

[d] Normal exposure level

[e] Negative control

[f] 50 Metaphase plates and 50 diakinesis/metaphase I spermatocytes scored per animal

APPENDIX III

Direct Bone Marrow Preparation
(modified after Tjio and Whang, 1965)

Solutions: Colcemid solution. A 0.3 μg/ml solution was made from 4 μg/ml colcemid lypholized in phosphate buffered saline (Grand Island Biological, Grand Island, IL).

Hypotonic solution. 1% sodium citrate in distilled water.

Alcohol-acetic acid fixative. 3 volumes methanol to 1 volume acetic acid.

Tissue preparation and fixation:

1. Both femurs were removed from each mouse. The distal end was cut off and the end freed of debris. The marrow was removed by flushing the shaft of each femur with 1.5 ml colcemid solution injected through the trochanteric fossa and collected in a 20 ml conical centrifuge tube. The marrow was left for 2-3 hours at room temperature (23°C) in the capped centrifuge tube.
2. The colcemid solution was removed by centrifuging the tube at room temperature at 400 rpm for 5 minutes. The supernatant was removed and 3 ml of hypotonic solution added. To break up cell clumps each tube was shaken. Hypotonic treatment lasted 30 minutes.

3. Again the tube was centrifuged at room temperature at 400 rpm for 5 minutes. The supernatant was removed and 5 ml of alcohol-acetic acid fixative was added. The tubes were agitated to resuspend the cells and fixed for 5 minutes. This procedure was repeated again.

4. The fixed suspension was stored at 0-4^{o}C and the slides were made after all experiments had been completed.

5. To prepare slides the bone marrow cell suspension was centrifuged for 5 minutes, all supernatant removed, and 0.5 ml fresh alcohol-acetic acid fixative was added. With a Pasteur pipette three evenly spaced drops of the cell suspension were put on a methanol-cleaned slide. The drops were blown gently to assist in spreading and drying of the preparation. The final beads of moisture left on the slide were shaken off. Slides were thoroughly dried before staining.

6. Thoroughly dried slides were coverslipped using Histoclad, a mounting medium (Clay-Adams, Parsippany, N.J.).

APPENDIX IV

Meredith Method of Male Meiotic Cell Preparation (Meredith, 1969)

Solutions: 1% sodium citrate in distilled water.

Alcohol-acetic acid fixative. 3 volumes ethanol to 1 volume acetic acid.

Tissue fixation and slide preparation:

1. Hypotonic treatment of the testis was begun when the testis was placed in 10 ml of 1% sodium citrate in a Petri dish, the tunica albuginea removed and the tubules were washed.
2. The washed tubules were then transferred to fresh 1% sodium citrate and gently unraveled with two fine-tipped dissecting needles. Care had to be taken not to rupture the tubules.
3. For mouse tubules the total hypotonic treatment time was 12 minutes.
4. The tubules were removed with a fine forceps and drained against a clean glass slide. The drained tubules were transferred directly to 20 ml of freshly made alcohol-acetic acid fixative. The fixed tubules were stored in the refrigerator at 0-4^{o}C until the slides were needed.
5. To prepare spermatocyte slides from fixed tubules a few milliliters of alcohol-acetic acid fixative was decanted into a Petri dish. Using fine-tipped dissecting needles, a 2-inch

equivalent of tubule was separated and transferred to 0.5 ml of 60% acetic acid in a small agglutination tube. The tube was tapped to suspend cells. Immediately a drop of cell suspension was transferred with a micropipette to a warmed (60°C) alcohol-cleaned glass slide. This drop was quickly withdrawn from the slide with the micropipette and expelled onto a different region of the same slide. This process was repeated until the slide surface had been covered. (Note: More than one slide may be made from the described suspension. However, cells begin disintegration after about 5 minutes in 60% acetic acid and Meredith recommends not using the suspension longer than 5 minutes.)

6. Slides were thoroughly dried before staining.

APPENDIX V

Wolbach Giemsa Staining
(modified after Lillie, 1965)

1. Thoroughly dried slides were stained 20 minutes in 1 ml Wolbach Giemsa stain, 2 ml Sorensen's phosphate buffer (pH 6 for bone marrow and pH 6.5 for spermatocytes) and 47 ml distilled water.
2. Slides were rinsed by repeated immersing and removal in Sorensen's buffer for 10-15 seconds.
3. The slides were finally rinsed with running tap water for 10 minutes.
4. Thoroughly dried slides were coverslipped using Histoclad.

APPENDIX VI

Methacrylate Embedding Procedure

1. McDowell-Trump's fixative was drained from the vials of fixed tissue and cacodylate buffer (0.2 M, pH 7.2 with 0.25 g $CaCl_2$/500 ml) was added. Tissues were kept in buffer overnight (16-24 hours) in the refrigerator.
2. The buffer was removed from the tissues. Tissue dehydration was carried out with the following series of alcohols: 50% ethanol; 70% ethanol; 95% ethanol; and three changes of 100% ethanol. Tissues were kept in each alcohol change 10-15 minutes.
3. The alcohol was removed and 2 ml of methacrylate solution "A" (glycol methacrylate, butoxyethanol, and benzoyl peroxide, Ivan Sorval, Inc., Newton, CT) was added. The vials were placed on a shaker unit overnight (16-24 hours).
4. The vials were drained and the tissue pieces were placed in individual Peel-A-Way embedding boats, sizes T-8 and T-12 (Peel-A-Way Scientific, South El Monte, CA). Approximately 7 ml of fresh methacrylate solution "A" was added.
5. The boats containing the tissues were exposed to γ-radiation from a ^{60}Co source for 20-30 minutes to catalyze the hardening of the embedding medium.

APPENDIX VII

Hematoxylin-Eosin Staining Procedure

Thoroughly dried slides were stained at 60°C with Delafield's hematoxylin (Carolina Biological Supply Co., Gladstone, OR) for 15 seconds and gently washed in water. Eosin, containing 10 g eosin "Y", 50 ml of distilled water and 940 ml of 95% ethanol, was placed on the warmed slide for 3-5 seconds. The slides were cover-slipped using Histoclad.