

The Effects of Mono-(2-ethylhexyl)-phthalate on Rat Sertoli Cell-Enriched Primary Cultures

ROBERT E. CHAPIN,¹ TIM J. B. GRAY,* JERRY L. PHELPS, AND SANDRA L. DUTTON

National Institute of Environmental Health Sciences, National Toxicology Program, P.O. Box 12233, Research Triangle Park, North Carolina 27709, and *British Industrial Biological Research Association, Woodmansterne Road, Carshalton, Surrey, SM5 4DS, England

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The Effects of Mono-(2-ethylhexyl)-phthalate on Rat Sertoli Cell-Enriched Primary Cultures. CHAPIN, R. E., GRAY, T. J. B., PHELPS, J. L., AND DUTTON, S. L. (1988). *Toxicol. Appl. Pharmacol.* **92**, 467-479. There is considerable evidence from *in vivo* studies that the Sertoli cell is an initial target cell for the actions of phthalates in the rodent testis. Because this metabolically active cell type plays a central role in spermatogenesis, we examined the effects of a toxic phthalate, mono-(2-ethylhexyl)-phthalate (MEHP), on the secretory and synthetic activities of primary testicular cell cultures isolated from 18-day-old rats. These cultures were 78-84% Sertoli cells. Exposure to MEHP decreased cellular ATP by ca. 20%, decreased production of radiolabeled $^{14}\text{CO}_2$ from acetate, and decreased media levels of pyruvate, while it increased media levels of lactate and intracellular lipid. Protein synthesis, evaluated by radiolabeled leucine incorporation, was not affected by MEHP. Mitochondrial succinate dehydrogenase activity was decreased in the presence of MEHP. Michaelis-Menton kinetic analysis indicated this was a mixed inhibition. There was no apparent change in mitochondrial Rhodamine 123 uptake. These data are consistent with the concept that mitochondria are one target for the actions of MEHP in the Sertoli cell. © 1988 Academic Press, Inc.

Esters of phthalic acid with specific chain lengths have been shown to be toxic to the liver (Lake *et al.*, 1975; Thomas and Thomas, 1984) and reproductive system (Gray *et al.*, 1977; Creasy *et al.*, 1983; Lamb *et al.*, 1987). In hepatocytes, phthalates have been shown to disturb a number of mitochondrial functions (Inouye *et al.*, 1978; Lake *et al.*, 1975; Takahashi, 1977; Shindo *et al.*, 1978). Di-(2-ethylhexyl)-phthalate is one phthalate which produces testicular atrophy (Foster *et al.*, 1980). One of the major metabolites *in vivo* is mono-(2-ethylhexyl)-phthalate (MEHP) (Albro *et al.*, 1973), which has been shown to produce the same spectrum of effects as that

produced by the parent compound (Lake *et al.*, 1975; Gray and Beaman, 1984).

Histologic and X-ray microprobe analysis have demonstrated that Sertoli cells and spermatids are the cells in the testis that first appear affected by the "active" phthalates (those phthalates that are toxic to the testis) (Foster *et al.*, 1982; Creasy *et al.*, 1983). The initial effects observed ultrastructurally involve dilation of the Sertoli cell smooth endoplasmic reticulum and mitochondrial condensation (Foster *et al.*, 1982; Creasy *et al.*, 1983).

The Sertoli cells in adult animals are non-dividing somatic cells that provide a permissive milieu for spermatogenesis. They are metabolically active and contribute to the formation of seminiferous tubule fluid (Waites and Gladwell, 1982). They are the

¹ Address reprint requests and correspondence to Robert E. Chapin, NIEHS, P.O. Box 12233, Mail Drop E2-01, Research Triangle Park, NC 27709.

source of numerous proteins (Wright *et al.*, 1981; Skinner *et al.*, 1984) and have been shown to contain and secrete androgen binding protein (Kierszenbaum *et al.*, 1980). *In vitro*, they secrete lactate and pyruvate, which support germ cell ATP production, protein synthesis, and oxygen consumption (Jutte *et al.*, 1981; Mita and Hall, 1982; Nakamura *et al.*, 1984).

The objectives of these studies were two-fold. Because of the known synthetic and secretory activities of the Sertoli cells (above), we wanted to examine the effects on these functions of a compound which is known to affect Sertoli cells *in vivo*. Additionally, we wanted to determine whether some of the effects of MEHP observed in hepatocytes were also produced in Sertoli cells. Because the Sertoli cells comprise less than 3% of the cells in the mature testis, fractionation of adult testes greatly dilutes the Sertoli-derived products with material from other cells. Cell culture provides a controlled, albeit somewhat artificial, alternative to the limitations of whole-testis fractionation. The data presented below suggest that Sertoli cells and hepatocytes differ slightly in their response to phthalates.

METHODS

Chemicals. MEHP was synthesized by the method of Albro *et al.* (1973), and its purity (>98%) established by HPLC, NMR spectroscopy, and elemental analysis. For use *in vitro*, MEHP was dissolved in dimethyl sulfoxide and added at 4 μ l/ml of culture medium. Eagle's culture medium with Earle's salts (MEM), Hanks' balanced salt solution (HBSS), and cycloheximide were obtained from GIBCO (Long Island, NY). All chemicals for the lumino-metric determination of ATP and ADP were obtained from LKB (Gaithersburg, MD). The ovine follicle stimulating hormone (FSH) and growth hormone (GH) were gifts of the National Hormone and Pituitary Distribution Program. Radiochemicals were purchased from ICN Radiochemicals (Irvine, CA), while [1^{-14}C]pyruvate was obtained from Amersham (Arlington Heights, IL). Rhodamine 123 was obtained from Kodak Chemical Co. (Rochester, NY). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Cultures. Litters of rats were reared by F-344 parents in the NIEHS breeding colony under conditions described

previously (Chapin *et al.*, 1987). Eighteen-day-old males were killed by asphyxiation with carbon dioxide, the testes were removed, and enriched Sertoli cell cultures were prepared as described (Chapin *et al.*, 1987), following the method of Kierszenbaum and Tres (1981). Briefly, decapsulated testes were minced and sequentially incubated with trypsin, then collagenase. Fragments of seminiferous tubules were separated from single cells by gravity sedimentation. The fragments were plated at confluent density in 75-cm² culture flasks (26-ml initial volume, 1.8–2.5 mg protein per flask for lactate, pyruvate, and ATP/ADP assays), 60-mm-diameter culture dishes (for protein synthesis; both from Falcon), or center-well flasks (Kontes No. K882360-0025, for $^{14}\text{CO}_2$ generation studies). Cells were maintained at 32°C in 5% CO₂/95% air in MEM without antibiotics or serum; the medium was supplemented with transferrin (5 $\mu\text{g}/\text{ml}$), insulin (5 $\mu\text{g}/\text{ml}$), retinol acetate (5 μM), testosterone and dihydrotestosterone (0.1 μM each), epidermal growth factor (3 ng/ml), and GH (6.5 $\mu\text{U}/\text{ml}$) (Tres and Kierszenbaum, 1983). Cultures were exposed to MEHP when confluent, the third day after plating. FSH was used in some experiments at a concentration of 0.1 $\mu\text{g}/\text{ml}$.

Culture composition. Greater than 2000 cells per isolation were counted to determine the composition of each isolation (Chapin *et al.*, 1987). The cultures consisted of 78–84% Sertoli cells (judged by staining for oil red O), 1.5–6.5% peritubular cells (positive staining for alkaline phosphatase), and the remainder (ca. 15%) were germ cells, as determined by nuclear morphology and their position in the culture atop the Sertoli layer.

For other histologic analyses, cells attached to cover-slips were fixed with 4% neutral buffered formalin for 10 min, rinsed with water, and stained with hematoxylin and oil red O by standard procedures (Luna, 1968).

Assays. Media lactate was assayed by the method of Maurer and Poppendiek (1974), using 3-acetylpyridine adenine dinucleotide (APAD) as cofactor. The method was modified to use 33% less APAD after first determining that this did not change the rate or endpoint of the reaction. Pyruvate in the medium was assayed by the method of Czok and Lamprecht (1974). At the times indicated under Results, 1 ml aliquots of media were removed from the culture flasks and centrifuged to remove any cells, and the cell-free supernatant was frozen in dry ice and stored at –80°C until assayed (less than 7 days after collection). At the end of the experiment, the layer of cells was dissolved in 10 ml 0.5 M NaOH, and the protein was determined by the method of Bradford (1976), using bovine serum albumin in NaOH as the standard. The data are expressed as micrograms/milliliter/milligram protein. Correcting for the volume of medium removed did not affect the trend or significance of the results.

For determination of cellular ATP, the media was removed and the cells were overlayed with 10 ml of 5% trichloroacetic acid (TCA) containing 2 mM ethylene-

diaminetetraacetic acid (EDTA) to inactivate phosphatases and extract the ATP. An aliquot of this was diluted 1:20 with 0.1 M Tris-acetate, pH 7.75, containing 2 mM EDTA, and either assayed fresh or frozen on dry ice and stored at -80°C for <5 days. Samples for medium ATP determination were centrifuged, frozen on dry ice, and stored at -80°C until assayed. ATP was assayed on an LKB 1251 Luminometer. The assay was linear from 10⁻⁷ M to 10⁻¹² M ATP; standards were made up in Tris-acetate (for cellular determinations) or MEM (for media levels); each sample was corrected for internal quenching. Cellular levels of ADP were determined by the method supplied by LKB, wherein ATP was first measured as above, then ADP was converted to ATP, using phosphoenolpyruvate and pyruvate kinase. The ADP assay was linear from 10⁻⁶ M to 10⁻⁹ M. After extraction of the cells with TCA/EDTA, the protein was dissolved in 0.5 M NaOH and assayed by the method of Bradford (1976). The nucleotide data are expressed as nanomoles/milligram protein.

Cellular protein synthesis was estimated by the method of Villa *et al.* (1980), using [³H]leucine or [³H]tyrosine. Synthesis and release of secreted proteins were assayed by adding 0.4 ml of a cold carrier solution (5 mg/ml L-leucine or tyrosine, 0.15 M NaCl, 1% BSA) to 4 ml of cell-free medium. After mixing, 4 ml of 10% TCA was added, mixed, and allowed to precipitate. The precipitate was pelleted by centrifugation, washed four times with 5% TCA (or until counts in the supernatant fell to background), and then dissolved in 500 µl of 0.1 M NaOH. Cells in the monolayer were digested with 0.5 M NaOH, and the protein was assayed by dye binding (Bradford, 1976). The data are expressed as dpm/µg cellular protein.

For generation of ¹⁴CO₂ from radiolabeled acetate, cells were plated in glass center-well flasks. On the third day after plating, the cells were dosed with medium containing MEHP. At various times after dosing (indicated under Results), the flasks were capped, and 1 µCi of [1,2-¹⁴C]acetate (sp act 40–60 mCi/mmol) was injected into the media. The flasks were maintained in a shaking waterbath (32°C, 60 cpm) for 1 hr, when the reaction was stopped by addition of 500 µl of 30% perchloric acid. The ¹⁴CO₂ was trapped in 350 µl of benzethonium hydroxide (1 M in methanol) for 2 hr at 32°C with shaking, after which the center well was placed in 5 ml Aquasol (DuPont) and counted in a Beckman 3800 scintillation counter. The monolayer was dissolved in 2.5 ml 0.5 N NaOH and assayed for protein by the method of Bradford (1976). The data are expressed as dpm/hr/µg protein.

Pyruvate dehydrogenase activity *in situ* was estimated by incubating 1 µCi [1-¹⁴C]pyruvic acid (10–30 Ci/mol) per center-well flask. The incubations and subsequent handling were as for ¹⁴CO₂ evolution from pyruvate (above), except that only 1 and 4 hr of exposure to MEHP were evaluated.

Succinate dehydrogenase (SDH) activity was measured in mitochondria isolated from Sertoli cells treated

in vitro. Cells were treated for 4 or 24 hr with MEHP, after which the medium was removed and mitochondria were isolated after the method of Stancliff *et al.* (1969). Two milliliters of 0.33 M sucrose, 5 mM Tris-HCl, 0.5 mM EDTA (pH 7.6) was added, and the cells were scraped off the plate with a rubber policeman and kept on ice for ca. 10 min. The cells were then sonicated for 10 sec and centrifuged at 750g for 10 min. The pellet was washed with 2 ml sucrose-Tris-EDTA and centrifuged again. The two supernatants for each tube were combined and centrifuged at 9000g for 10 min. The pellet was washed with sucrose-Tris-EDTA once, resuspended in 1 ml of 0.2 M potassium phosphate buffer, and kept on ice until assayed. SDH activity was assayed by the method of Pennington (1961). The assay was linear with time and protein; the data are expressed as micromoles/minute/milligram protein. When SDH activity was evaluated in the presence of MEHP, MEHP was added to the isolation and assay buffers in the same concentration as had been used to dose the cells. These experiments were performed three times; representative data from one experiment are shown.

Rhodamine 123 (Rh123) staining. Cells to be stained with Rh123 were plated on glass 22 × 22-mm coverslips and maintained and dosed as above. At 4, 8, or 24 hr after dosing, the MEHP-containing medium was removed and replaced with MEM containing MEHP and 5 µg Rh123/ml. Cells were incubated with this medium in the dark for 10 min, after which they were rinsed once through MEM without Rh123 and mounted with a drop of MEM on a glass slide. The coverslips were examined by two observers uninformed of the treatment of each coverslip.

Statistics. All the biochemical and morphologic experiments above were done with four to seven flasks per group and were repeated at least three times. However, for clarity, data from one replicate are reported. Differences between groups were identified by analysis of variance, and the significance of the difference between group means was assessed with Student's *t* test. Differences in slope of kinetics data were analyzed using the SAS software package (SAS Institute; Cary, NC). For all tests, the significance level was set at *p* < 0.05.

RESULTS

There were concentration-related changes in the appearance of the cells in culture (Fig. 1); increasing concentrations of MEHP increased the neutral lipid content of the Sertoli cells, as judged by staining with oil red O. Greater MEHP concentrations also caused the cells to retract into mounds of cells, producing a discontinuous monolayer. There was an ap-

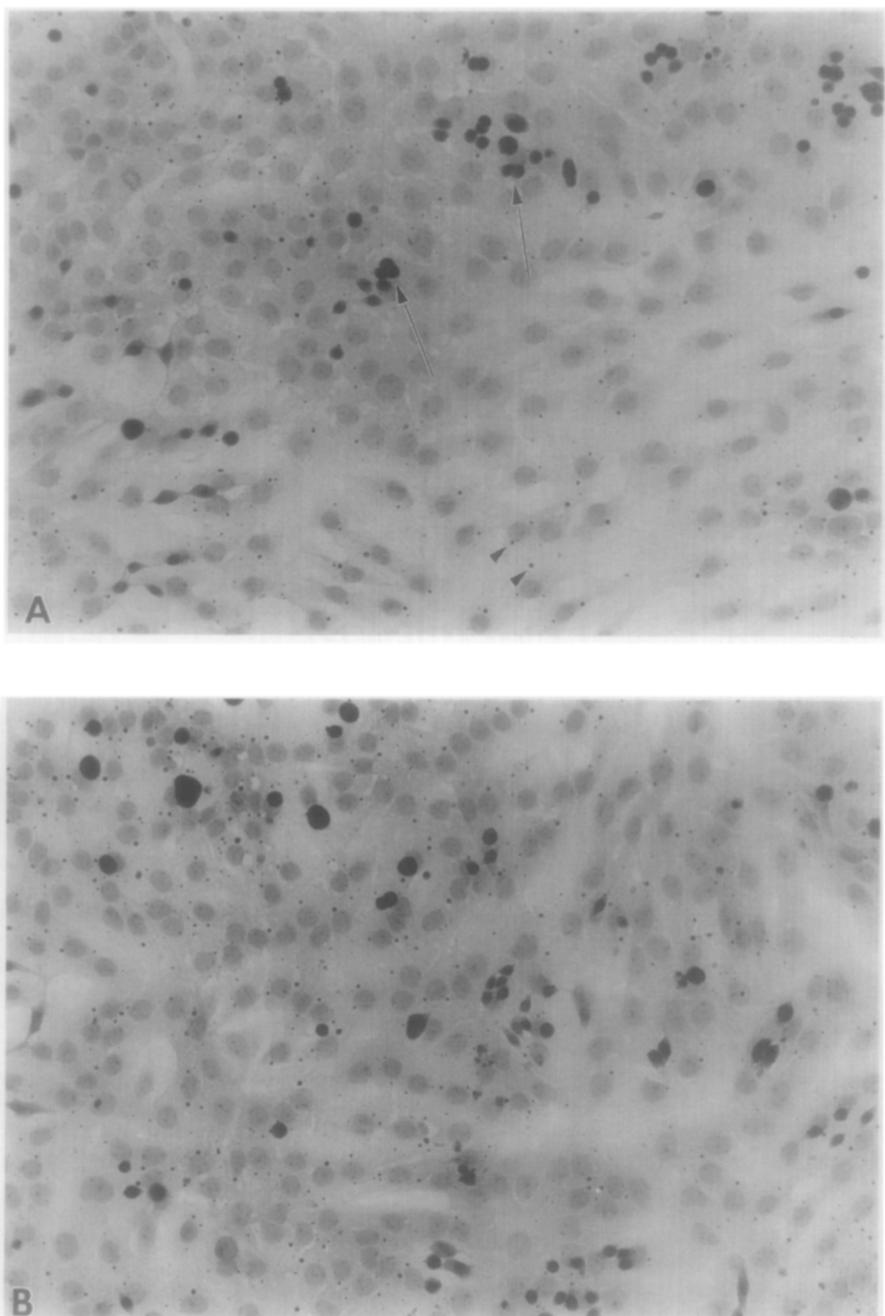


FIG. 1. (A) Sertoli cell-enriched cultures, maintained *in vitro* for 96 hr, stained with hematoxylin and oil red O. The germ cells (arrows) and lipid droplets (arrowheads) are readily visible. (B-F) Cultures after 24-hr exposure to 0.003 mM (B), 0.01 mM (C), 0.03 mM (D), 0.10 mM (E), and 0.30 mM (F) MEHP. With increasing concentration, the lipid droplets become more prominent and the monolayer is less confluent. [All 230X.]

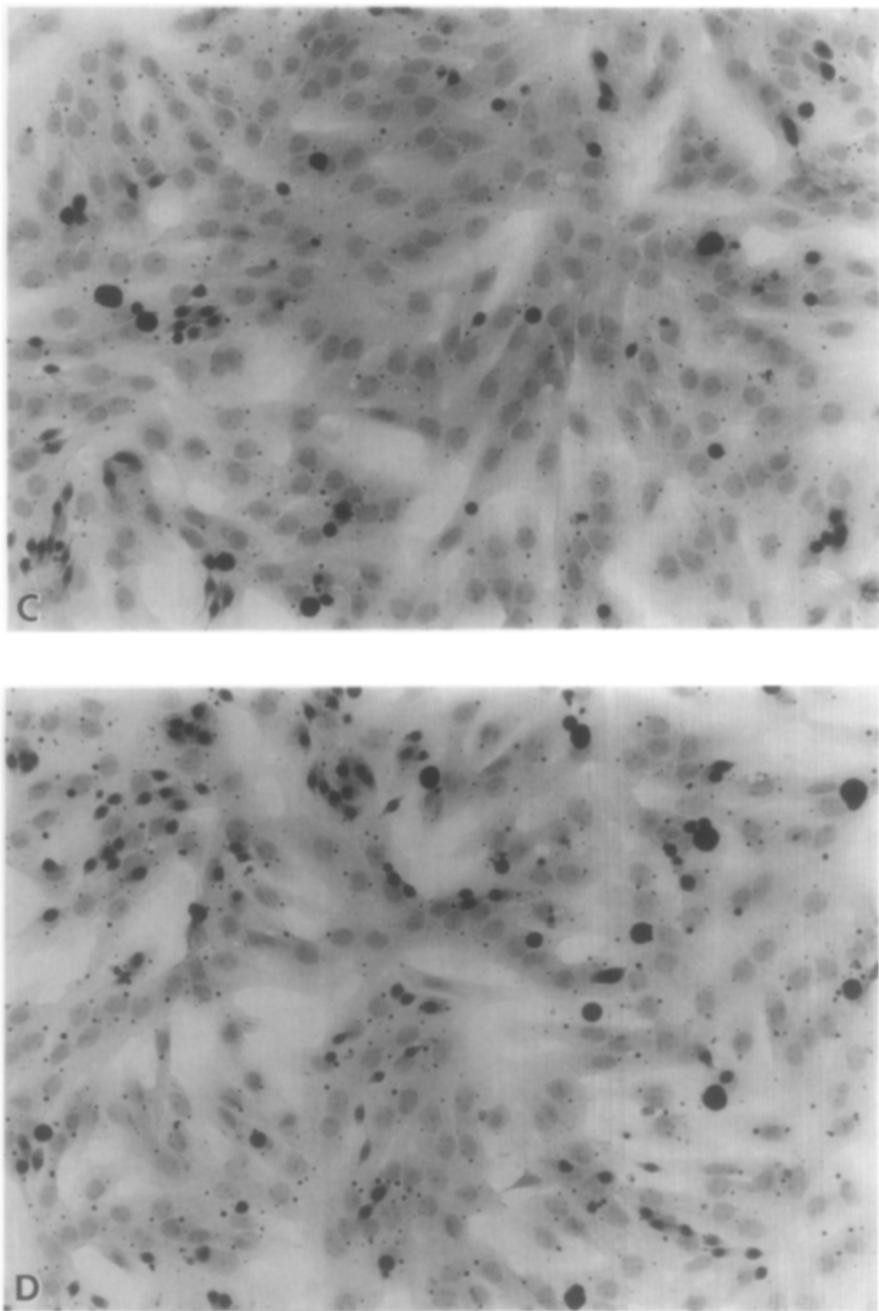


FIG. 1—Continued.

parent decrease in the number of germ cells atop the Sertoli cell layer, but no increase in the number of dead or dying Sertoli cells, as assessed by nuclear pyknosis or chromatolysis in the hematoxylin-stained cells. The germ cell

decrease was qualitatively similar to that reported by Gray and Gangolli (1986) for testicular mixed-cell cultures *in vitro*.

MEHP exposure produced time- and concentration-dependent increases in the

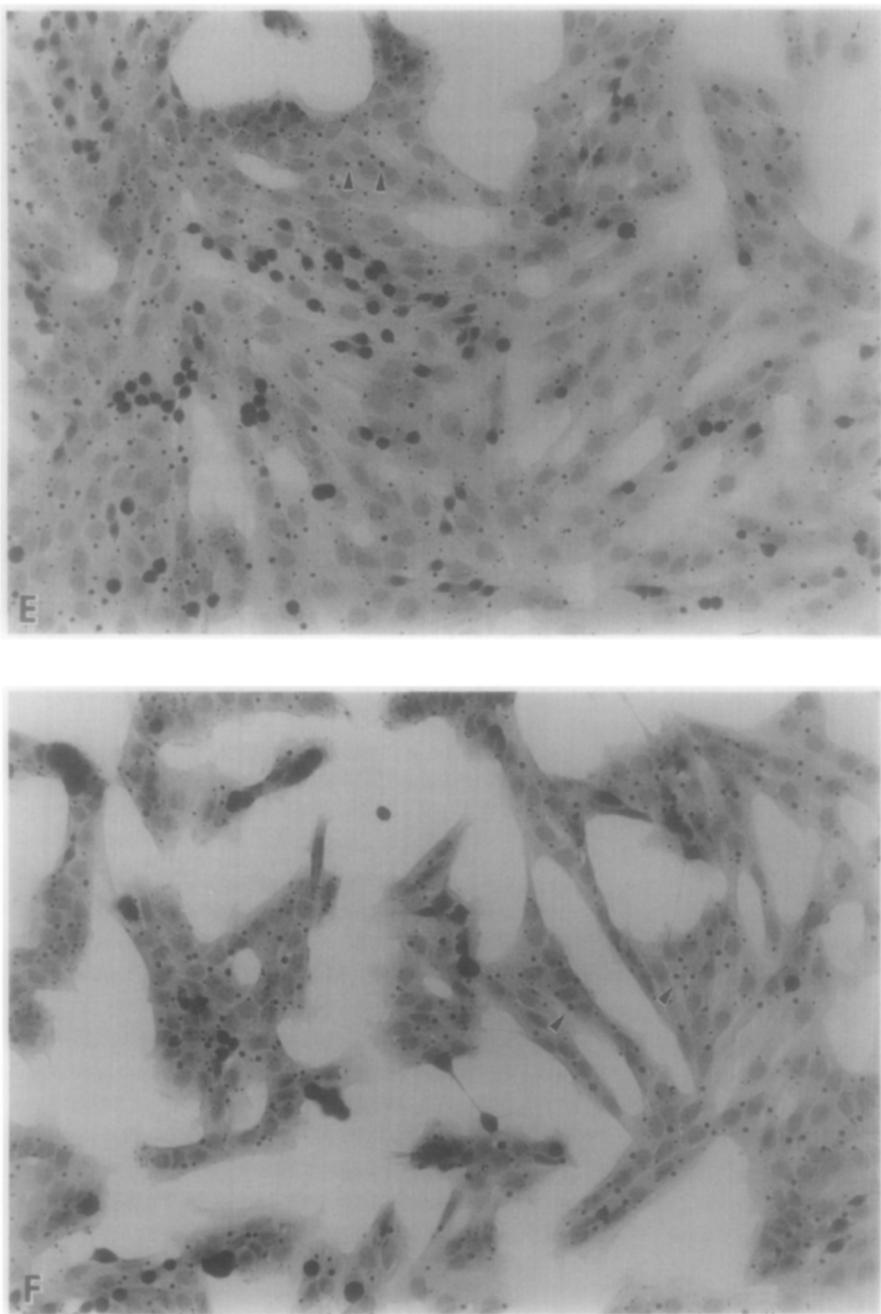


FIG. 1—Continued.

amount of lactate in the medium, but decreased the pyruvate (Fig. 2). All replicate experiments had a threshold concentration of 0.01 mM MEHP for increasing lactate, while media pyruvate was affected at 0.003 mM

MEHP. The presence of FSH in the medium before or during exposure did not affect the response to MEHP (not shown).

MEHP did not affect the amount of tritiated leucine incorporated into acid-precipita-

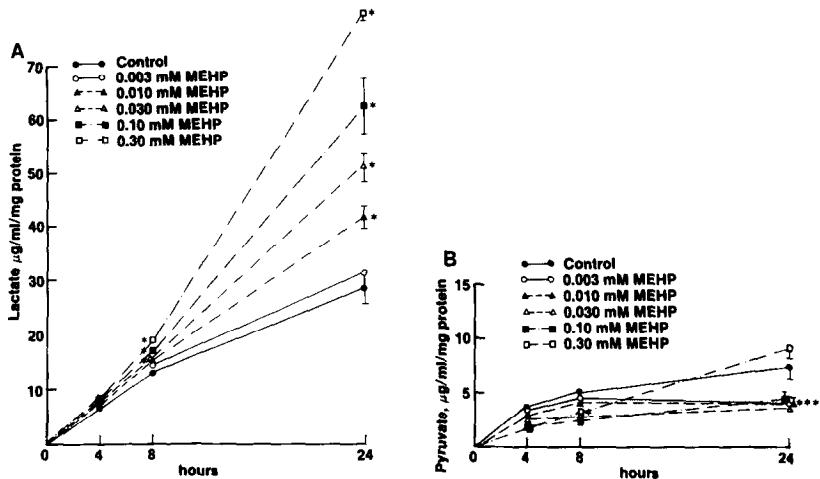


FIG. 2. (A and B) Concentrations of lactate and pyruvate in the medium during exposure to MEHP, mean \pm SD, expressed as micrograms/milliliter/milligram cellular protein. Notice that while lactate increases with concentration and time, pyruvate is decreased by all but the highest concentration of MEHP. $n = 4$ /group. *Significantly different from control.

ble protein from either the cellular monolayer or protein secreted into the medium (Table 1). In these same experiments, a group containing 2.5 μ M cycloheximide was included. Cycloheximide decreased leucine incor-

poration by >90% (not shown). Similar data were obtained using tyrosine as the labeled amino acid (not shown). This was corroborated by the cellular protein measurements made for the lactate and pyruvate experiments; there were no consistent changes in the amount of protein present in those experiments.

After 24-hr exposure to MEHP, levels of ATP were decreased in the cells by 15–20% (Table 2); ADP was unaffected. The thresh-

TABLE 1

INCORPORATION OF [3 H]LEUCINE INTO ACID PRECIPITABLE PROTEIN DURING MEHP EXPOSURE^a

Dose (mM)	24 hr
Cells	
0.0	28.7 \pm 4.9
0.003	32.6 \pm 3.1
0.030	29.9 \pm 4.7
0.30	31.0 \pm 3.3
Medium	
0.0	13.3 \pm .09
0.003	12.0 \pm 2.7
0.010	13.4 \pm 0.9
0.03	11.9 \pm 1.5
0.10	10.5 \pm 1.7
0.30	13.1 \pm 0.3

^a Values are expressed as means \pm SD, dpm/ μ g cellular protein, $n = 5$. The incorporation times were 1 hr for cellular protein, and 24 hr for proteins secreted into the medium.

TABLE 2

CELLULAR ATP AND ADP LEVELS AFTER 24-HR MEHP EXPOSURE^a

Dose (mM)	ATP	ADP
0.0	60.54 \pm 3.01 \times 10 ⁻⁹	11.91 \pm 1.34 \times 10 ⁻⁹
0.003	60.36 \pm 0.50 \times 10 ⁻⁹	10.94 \pm 1.29 \times 10 ⁻⁹
0.01	52.47 \pm 2.52 \times 10 ^{-9*}	12.02 \pm 0.26 \times 10 ⁻⁹
0.03	49.87 \pm 2.24 \times 10 ^{-9*}	9.22 \pm 0.62 \times 10 ^{-9*}
0.10	51.94 \pm 0.64 \times 10 ^{-9*}	11.61 \pm 0.70 \times 10 ⁻⁹
0.30	49.70 \pm 2.20 \times 10 ^{-9*}	10.94 \pm 0.54 \times 10 ⁻⁹

^a Values are expressed as means \pm SD, moles/milligram cellular protein, $n = 4$.

* Significantly different from control.

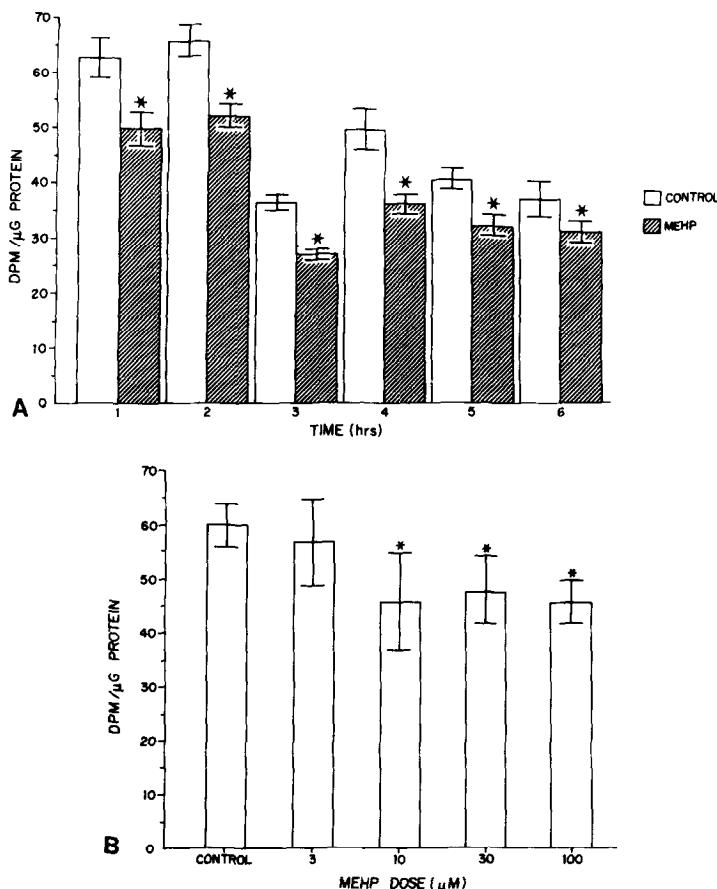


FIG. 3. (A) Time course of $^{14}\text{CO}_2$ production from $[1,2-^{14}\text{C}]$ acetate during exposure to 0.1 mM MEHP. Values are expressed as the mean \pm SD, dpm's per minute per milligram cellular protein, $n = 6$. *Significantly different from control. (B) Generation of $^{14}\text{CO}_2$ from $[1,2-^{14}\text{C}]$ acetate after 4 hr of exposure to different concentrations of MEHP. Values expressed as above.

old concentration for this effect was 0.01 mM MEHP. Effects on ATP were delayed relative to changes in lactate and pyruvate, for there was no change in ATP at 1, 4, or 8 hr. Levels of ATP or ADP in the medium, which might be expected to increase in the presence of cell membrane damage, were not affected by MEHP exposure (not shown).

Since the evidence of increased glycolysis (media lactate levels) and decreased ATP suggested a block in Krebs cycle or electron transport, we examined the activity of the Krebs cycle by evaluating the effect of MEHP on $^{14}\text{CO}_2$ production from $[1,2-^{14}\text{C}]$ acetate. Exposure to 0.1 mM MEHP for as little as 1 hr decreased

$^{14}\text{CO}_2$ production (Fig. 3A); this decrease was maintained for up to 24 hr (not shown). This response, like the ATP and pyruvate endpoints, gave a plateau-type effect; concentrations greater than the minimal effective level gave no increased effect (Fig. 3B). When $[1-^{14}\text{C}]$ pyruvic acid was used as the substrate for $^{14}\text{CO}_2$ evolution, MEHP had no effect after 4 hr of treatment with MEHP (485 \pm 84 dpm/hr/ μg protein for controls; 520 \pm 79 dpm/hr/ μg protein for 100 μM MEHP; mean \pm SD, $n = 5$). There was no effect at 1 hr or at other doses of MEHP (not shown).

Because previous reports indicated that SDH in hepatic mitochondria was affected by

TABLE 3
SUCCINATE DEHYDROGENASE ACTIVITY^a

MEHP (mM)	4 hr	24 hr
Control	9.00 ± 1.67	14.92 ± 1.11
0.003	7.89 ± 1.32	13.87 ± 0.63
0.01	8.33 ± 1.93	14.16 ± 0.70
0.03	10.37 ± 1.22	15.22 ± 1.12
0.10	11.99 ± 0.86*	19.16 ± 2.32*

^a Activity is measured in the absence of MEHP in mitochondria isolated from Sertoli-enriched cultures treated with various concentrations of MEHP for 4 hr. Values are the means ± SD of micromoles product formed/minute/milligram cellular protein, *n* = 6.

* Significantly different from control.

MEHP, we examined SDH activity in mitochondria from MEHP-treated Sertoli cell-enriched cultures. Table 3 shows that when these cells were exposed to MEHP for 4 hr and mitochondria were isolated in the *absence* of MEHP, there was no decrease in SDH activity, nor was a decrease noted at any concentration after 24-hr exposure to MEHP. Rather, there was an increase in SDH activity. This suggested a compensatory increase, so we added MEHP to the SDH assay buffer at the same concentration present in the cell culture medium and found a dose-related decrease in SDH activity (Table 4). Double-reciprocal plots showed that both $1/V_{max}$ and $-1/K_m$ changed upon addition of MEHP, suggesting a mixed inhibition (Fig. 4).

There was no visible difference in fluorescent intensity of Rh123 staining between control cells and MEHP-treated cells, even at concentrations and times (0.1 mM, 24 hr) which produced marked morphologic changes in the cells. Although at these times the distribution of the mitochondria was different due to the changes in cell shape, there was no observable decrease in the intensity of the staining.

DISCUSSION

The studies reported above detail a series of effects which occur after exposure to MEHP.

The time-course studies showed a relatively quick (<60 min) decrease in the conversion of [¹⁴C]acetate to ¹⁴CO₂. The effects on glycolysis took longer to become distinct, though by 4 hr, levels of media pyruvate had started to become lower than controls, and a trend in lactate levels emerged. This increase in glycolysis, manifest as increased media lactate, apparently maintained cellular ATP levels for a time, for these ATP levels had not declined after 8-hr exposure, though they were decreased at 24 hr. One hypothesis is that the lactate resulting from this increased glycolysis was extruded from the cell, while the pyruvate, whose utilization through the Krebs cycle was slowed, was used for lipid synthesis and was subsequently visualized as oil red O-positive droplets in the stained coverslips (Fig. 1). This is consistent with most of the above data, with the exception of the [¹⁴C]pyruvic acid ¹⁴CO₂ generation experiment. The lack of effect of MEHP on the production of ¹⁴CO₂ from pyruvic acid might be due to a change in the size of the pyruvate pool in the cells, or to other unaddressed events. Nonetheless, the decrease in SDH activity in the presence of MEHP is consistent with the interpretation that the Krebs cycle is at least one of the biochemical targets for MEHP in Sertoli cells.

A number of authors have described effects of phthalate esters on mitochondrial function

TABLE 4
SUCCINATE DEHYDROGENASE ACTIVITY WITH MEHP^a

MEHP (mM)	4 hr	24 hr
Control	18.7 ± 2.63	12.6 ± 2.64
0.003	13.9 ± 1.04*	11.5 ± 1.03
0.01	13.3 ± 3.36	11.2 ± 0.96
0.03	13.5 ± 3.32*	9.44 ± 0.62
0.10	10.0 ± 0.74*	8.32 ± 0.91*

^a Mitochondria were isolated from Sertoli-enriched cultures exposed to MEHP *in vitro* and during isolation. Values are means ± SD, micromoles product formed/minute/milligram protein, *n* = 6.

* Significantly different from control.

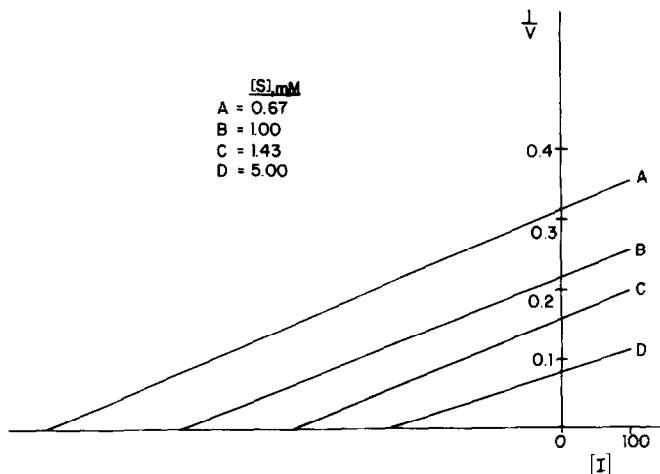


FIG. 4. Dixon plot ($1/V$ vs $[I]$ at different $[S]$) of the inhibition of SDH activity by MEHP. The slopes are not significantly different ($p > 0.1$).

in hepatocytes. This has been reported both after dosing of animals (Lake *et al.*, 1975; Srivastava *et al.*, 1975; Shindo *et al.*, 1978) and after *in vitro* incubation of isolated mitochondria (Melnick and Schiller, 1985; Inouye *et al.*, 1978; Ohyama, 1977). A common conclusion of these reports is that the "active" monoester phthalates inhibit oxidation of mitochondrial substrates, or act as weak uncouplers. These studies have routinely used millimolar concentrations of phthalates, with some exceptions (Melnick and Schiller, 1985; Inouye *et al.*, 1978). It is noteworthy that the effects seen above with the Sertoli cells were seen at concentrations as low as 0.01 mM. With a concentration of 0.1 mM, Melnick and Schiller saw effects on SDH that were of the same magnitude (~20% decrease) as were seen in the $^{14}\text{CO}_2$ -generation experiments, using Sertoli cell-enriched cultures.

Interestingly, one effect noted in hepatic mitochondria was not evident in our studies. Inouye *et al.* (1978) and Melnick and Schiller (1985) observed large decreases in the membrane potential of hepatic mitochondria incubated *in vitro* with MEHP. Because the amount of Rh123 taken up by organelles depends on the transmembrane potential of

those organelles (Johnson *et al.*, 1981), we expected to find a visible decrease in the Rh123 fluorescent signal from mitochondria of MEHP-exposed cells. Even though the uncoupler CCCP decreased Sertoli mitochondrial fluorescence to background levels (data not shown), there was no observable difference between MEHP-treated cells and DM-SO-treated control cells. It is easily conceivable that a 20% decrease in this potential, indicated by Rh123 fluorescence, would be invisible to the human eye. Nevertheless, the massive loss of membrane potential reported by Inouye *et al.* (1978) was clearly not present in Sertoli cell mitochondria.

Additionally, we found decreases in mitochondrial succinate dehydrogenase activity only in the presence of MEHP. Incubation of the cells with MEHP, followed by mitochondrial isolation, and SDH assay in the absence of MEHP produced an increase in SDH. The kinetics observed for SDH in the presence of MEHP are reminiscent of those proposed by Friedenwald and Maengwyn-Davies (1954) for the case where an inhibitor couples with the enzyme-substrate complex and not with the free enzyme. How this mode of action would explain the compensatory increase is not yet clear.

Creasy *et al.* (1983) report histochemical evidence of a loss of SDH activity in Sertoli mitochondria *in vivo*. SDH activity has been reported to be decreased in hepatic mitochondria exposed to DEHP or MEHP either *in vivo* (Lake *et al.*, 1975) or *in vitro* (Melnick and Schiller, 1985). Data from Melnick and Schiller (1985) support a competitive mode of inhibition. The cause for the differences between the hepatic and Sertoli cell SDH effects may be related to the different "microenvironments" and metabolic demands experienced by the two tissues and cell types.

An additional observation is that hepatic mitochondria swell when exposed to specific phthalate esters (Lake *et al.*, 1975; Ohyama, 1977; Melnick and Schiller, 1985). The literature on Sertoli mitochondria are conflicting. Foster *et al.* (1982) reported mitochondrial condensation after phthalate exposure *in vivo*. However, other authors found Sertoli mitochondrial hypertrophy after phthalate exposure, both *in vivo* (Creasy *et al.*, 1987) and *in vitro* (Creasy *et al.*, 1983). This hypertrophy appeared distinct from swelling; the latter is characterized by a decreased density of the internal structure, which was clearly not present in the MEHP-induced hypertrophy (Creasy *et al.*, 1983). Both the SDH data and the ultrastructural evidence in the literature suggest that hepatic mitochondria react to MEHP differently than do Sertoli cell mitochondria.

A paper by Reyes *et al.* (1986) reported experiments on the effects of gossypol on cultured TM4 cells, a line derived from mouse Sertoli cells. The increased lactate output from gossypol-treated TM4 cells was similar to that reported above for MEHP. Reyes *et al.* concluded from their experiments that gossypol acts as an uncoupler in these cells. However, the findings that (1) gossypol diminished Rh123 uptake by Sertoli mitochondria *in vitro* (Tanphaichitr *et al.*, 1983) and (2) that the lesion produced by gossypol in the testis is somewhat different from that produced by the phthalates (Hoffer, 1983) suggest that MEHP and gossypol may have

different mechanisms, even though they apparently share a target organelle.

The Sertoli cell holds a pivotal place in spermatogenesis; without normal Sertoli cell function, complete spermatogenesis cannot occur. Indeed, impaired Sertoli cell function is frequently invoked as a cause of germ cell loss, although there are no specific Sertoli cell functions whose impairment has been shown to result in germ cell death. Because the germ cells *in vitro* influence Sertoli cell function (D'Agostino *et al.*, 1984; Galdieri *et al.*, 1984; Jegou *et al.*, 1986; Welsh *et al.*, 1985) and may take up the lactate and pyruvate secreted by the Sertoli cells (Jutte *et al.*, 1981), a toxicant-induced decrease in the production of these small organics has been postulated as a cause of germ cell loss during alkoxy acid exposure (Beattie *et al.*, 1984). It is noteworthy that MEHP caused only a small decrease in pyruvate secretion *in vitro* and a large increase in lactate; thus, this theory cannot apply to the lesion produced by MEHP. Other patterns of changes have been reported for cadmium, lead, and methoxyacetic acid treatment of Sertoli cells *in vitro* (Clough *et al.*, 1986; Batarseh *et al.*, 1986; Pavittranon *et al.*, 1986, respectively). How closely the *in vitro* metabolic changes mimic the *in vivo* situation is, so far, unknown.

We observed no change in the synthesis of either secreted or cellular proteins. Preliminary evidence (not shown) suggests that secretion of androgen binding protein (ABP) *in vitro* is not affected by MEHP. This is consistent with Sertoli cells cultured under other conditions (Gray, personal communication), although *in vivo* exposure to toxic phthalates dramatically reduces *in vivo* ABP secretion (Gray and Gangolli, 1986). This is unlike the previously demonstrated concordance between the *in vivo* and *in vitro* situations (Gray, 1986); the reason for this variance is unclear.

In summary, MEHP exposure produced effects in Sertoli cell-enriched cultures that were consistent with a mild effect on the mitochondria of these cells, at concentrations

that were significantly lower than those reported by previous authors. The early effects on mitochondrial activity were followed by morphologic changes in the monolayer, although necrotic Sertoli cells were not seen over the times examined here. The changes in Krebs cycle activity appeared consistent with mixed-type inhibition of succinate dehydrogenase. Whether this is the principal target for producing toxicity in these cells and the response of cells from older animals remains the subject of further investigations.

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