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H19年度分担研究報告書

プラスティック製医療機器の安全性に関する研究
－フタル酸エステルDEHPとその活性代謝産物 MEHP の比較毒性学的研究－

文献や国際動向に関する調査研究によるMEHPとDEHPの比較研究

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研究要旨　　毒性評価が遅れているMEHPによる毒性プロファイルを明らかにするとともに、その内容を当班で行う実験プロトコール作成に役立てることを目的に、MEHPの毒性に関する内外の情報を特に、精巣、腎、神経、心臓毒性を中心に調べた結果、MEHPがPPARαのみならずPPARγのアゴニストであることが判明したが、PPARが精巣、腎、神経、心臓毒性に関与している明らかな証拠は無かった。一方で、ミトコンドリア、Na+-K+-ATPase、cAMP、アセチルコリン受容体等への影響が報告されていることから、これらのPPAR非依存的な作用にも注意する必要があると思われた。

A．研究目的

これまで医療用具等から溶出することが報告されていなかったMEHPが、最近になって、ガンマ線滅菌することによりプラスティック製医療器具等から溶出してくることが報告されたため、このMEHPの毒性プロファイルの把握する必要がある。そこで、MEHPによる毒性プロファイルを明らかにするとともに、その内容を当班で行う実験プロトコール作成に役立てることを目的に、MEHPの毒性に関する内外の情報を収集し整理する。

B．　研究方法

本年度は、MEHPの一般毒性、生殖毒性、薬理学的研究、毒性発現メカニズム研究等に関する直近までの文献の調査を継続して実施するとともに、標的臓器であることが示されている精巣、心臓、神経毒性、腎毒性に焦点をあてて情報収集を行った。

C，D．結果と考察

1)精巣毒性

*In vitro*の系でラットのセルトリ細胞初代培養に対するMEHPの影響が調べられている。18日齢のF-344ラット雄から得たセルトリ細胞（78-84%）にMEHPを添加すると細胞ATPのレベルが減少し、培地の乳酸、細胞内脂質が増加した。蛋白合成については影響は認められなかった。ミトコンドリアのコハク酸脱水素酵素は減少した(添加4時間後、3μMのMEHPで有意に減少)。この結果、ミトコンドリアがセルトリ細胞でMEHP標的の一つであることを示された(Chapin RE et al., 1988)。

これに関連して、CDラット肝から得られたミトコンドリアに対する影響がMEHPで調べられている。MEHPはコハク酸脱水素酵素活性を非競合的に阻害した(ki=2.1x10-4M)。さらに、脱共役作用を示した。一方、同時に調べられたDEHPでは1mMの濃度でもこれらの作用は見られなかった(Melnick RL et al., 1985)。

ラットセルトリ細胞の機能に対するMEHPの影響がさらに調べられている。F-344ラットから調整されたセルトリ細胞のFSH-刺激によるcAMPの蓄積をMEHPが阻害した(ID50; 2-9μM)。またMEHPは乳酸の分泌を促進した(ED50; 4-12μM)。これらの二つの作用の濃度レベルは同様であった。なお、ATPレベルの減少がMEHPによりセルトリ細胞で見られたが、28日齢以上のラットから得られたセルトリ細胞では見られなかったことから、ATP減少は、in vivoでのDEHPの作用機序で重要な部分ではないと推測している(Heindel J.and Powell CJ, 1992)。

28日齢ウィスターラットにMEHPを単回強制経口投与し、3、6、12時間後に精巣について検索した。Germ cellの分離と脱皮が観察され、セルトリ細胞内では中間フィラメントのビメンチンが崩壊した。アンドロジェン受容体の分布には変化は無かった。Testosterone-repressed-prostatic message-2遺伝子の発現が、3時間後に増加したが、6時間後に元のレベルに戻った。カスパーゼ-3活性が3と12時間で増加した。これらの増加はアポトーシスと関係しておらず、感度の高い初期マーカーして有用であるとしている(Dalgaard M et al., 2001)。

精巣でMEHPにより影響を受ける遺伝子がマイクロアレイを用いて検索されている。米国CIITのグループは雄F344ラット28日齢にMEHPを1000mg/kgの用量で単回経口投与後、1、2、3、6、12時間後に精巣のマイクロアレイ解析をAffymetrix社のchipを用いて実施した。最も、顕著に増加したのはThbs1（thrombospondin1）であった。その他、転写因子Nr0b1、ステロイド合成遺伝子（Cyp17a1、StAR)、コレステロール代

謝遺伝子(Dhcr7)が用量依存的に変化した(Lahousse SA et al., 2006)。

2)腎障害

MEHPの腎障害に関連する論文として*in vitro*の実験が行われている。ドイツのRothenbacherらはDEHPの代謝物が尿中に多く排泄されるのに、腎臓への影響が着目されていないことから近位尿細管由来のOK細胞(Oppossum (袋鼠) kidney epithelial cell)にMEHPあるいは2-EHPを0.1-500μMの濃度で3日間添加した。MEHPは用量に依存した細胞生存率減少をおこしたが(ED50=25μM)、2-EHPでは変化はなかった。またMEHPは細胞骨格線維のF-actinを減少させた(Rothenbacher K et al., 1998)。

上記以外に、MEHPと腎障害に関する論文は見られなかった。一方、DEHPと腎障害に関する論文は認められた。
B6C3F1マウスにDEHPを6000ppmの濃度で飼料に添加し、104週間投与すると雌雄に、1500ppmで雌に慢性進行性ネフロパチーが見られている(David RM et al., 2000)。

DEHPの腎障害についてPPARαノックアウトマウスを用いて影響が調べられている。DEHPを0.05または0.01%の濃度で資料に添加し、22ヶ月投与するとPPARαノックアウトマウスで糸球体腎炎が見られたことから、PPARαは腎障害に対してむしろ防御的に働いていることが示唆されている(Kamijo Y. et al., 2007)。

MEHPはPPARαのアゴニストであることは知られているが、最近MEHPがPPARγを直接活性化することが報告されている(Feige JN et al., 2007)。

PPARと腎障害に関する研究がin vitroで行われているPPARαのアゴニストとして、WY14623とクロフィブレートが、PPARγのアゴニストとして、pioglitazoneとciglitazoneが用いられ、3つの異なる近位尿細管細胞に対する毒性が調べられている。その結果、clofibrateとpioglitazoneには100μMの濃度まで細胞毒性は見られなかった。一方、WY14643とciglitazoneは細胞死を増加させることから、細胞死はPPARの活性化とは関係しないだろうと推測している(Giral H et al., 2007)。

MEHPによる腎障害の機序については、現時点では明らかでない。ミトコンドリアの酵素阻害があることから尿細管のATP合成阻害が生じて尿細管の機能が障害されているかもしれない。DEHPに関してはNa(+)-K+-ATPaseを阻害することが報告されている。詳細は明らかでないが、7.5mg/kgのDEHPをラット腹腔内投与すると脳、肝、RBCのNa(+)-K+-ATPaseが阻害された(Dhanya CR et al., 2003)。Na(+)-K+-ATPase

の阻害による腎障害についてアミノ配糖体抗生剤（ゲンタマイシン、ストレプトマイシン等）が良く知られている。従って、MEHPが尿細管のNa(+)-K+-ATPaseを阻害して腎障害を起こす可能性も候補の一つにあげられるであろう。いずれにしてもMEHPの腎障害の機序についてはさらなる研究が必要である。

3)神経毒性

MEHPの急性毒性試験での痙攣誘発作用、in vitroの系でのコリン作動性作用からMEHPの脳、神経系に対する影響が示唆され、文献検索を行ったが、MEHPの脳に対する影響を調べた文献は見つからなかった。一方、MEHPがトリプトファン経路の酵素であるα-amino-β-carboxymuconate-e-semialdehyde decarboxylaseを阻害することにより神経毒性を有するキノリン酸の産生を促進するとの報告がある。ただし、ラットにおいてMEHPやDEHP投与で尿中のキノリン酸の排出量は増えているが、神経毒性は特に認められていないので(Fukuwatari T et al., 2004)、これによりMEHPの神経毒性が引き起こされた可能性は低いと思われる。

4)心毒性について

心毒性についての新たな情報は無かった。心筋では無いが、in vitroで摘出ラット胃の筋肉を用いてPGE2やアセチルコリンによる収縮作用がMEHPにより減少したとの報告がある(Tavares et al., 1985)。

E．まとめ

MEHPの毒性試験の文献調査を精巣、腎、神経、心臓毒性を中心に調べた結果、MEHPがPPARαのみならずPPARγのアゴニストであることが判明した。しかし、PPARが精巣、腎、神経、心臓毒性に関与している明らかな証拠は無かった。一方で、ミトコンドリア、cAMP、Na+-K+-ATPase、アセチルコリン受容体等への影響が報告されていることから、これらのPPAR非依存的な作用にも注意する必要があると思われた。

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F. 健康危険情報

なし

G. 研究発表

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H. 知的所有権の出願・取得状況

1) 特許取得

該当なし

2) 実用新案登録

該当なし

3) その他

該当なし

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

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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 $^{14}CO_2$ 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.

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 Beamand, 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 nondividing 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

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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 twofold. 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 μl/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 luminometric 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-cm^2 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 $^{14}CO_2$ generation studies). Cells were maintained at 32°C in 5% CO_2/95% air in MEM without antibiotics or serum; the medium was supplemented with transferrin (5 μg/ml), insulin (5 μg/ml), retinol acetate (5 μM), testosterone and dihydrotestosterone (0.1 μM each), epidermal growth factor (3 ng/ml), and GH (6.5 μU/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 μg/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 coverslips 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 [^{3}H]leucine or [^{3}H]-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 $^{14}CO_2$ 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-^{14}C]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 $^{14}CO_2$ 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-^{14}C]pyruvic acid (10–30 Ci/mol) per center-well flask. The incubations and subsequent handling were as for $^{14}CO_2$ 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 750*g* 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 9000*g* 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-

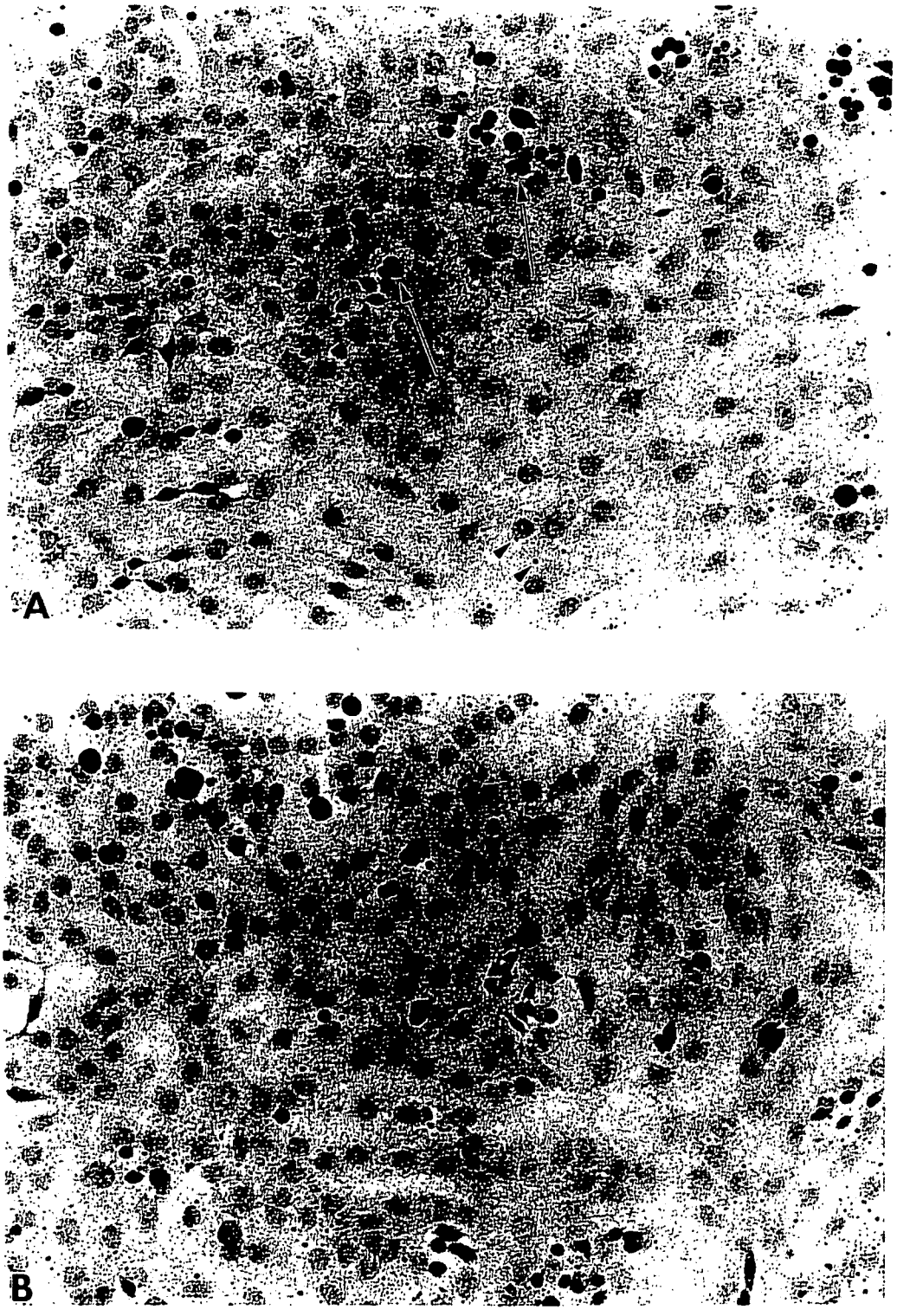

FIG. 1. (A) Sertoli cell-enriched cultures, maintained *in vitro* for 96 hr, stained with hematoxylin and oil red O. The germ cells (arrows) and lipid droplets (arrowheads) are readily visible. (B–F) Cultures after 24-hr exposure to 0.003 mM (B), 0.01 mM (C), 0.03 mM (D), 0.10 mM (E), and 0.30 mM (F)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

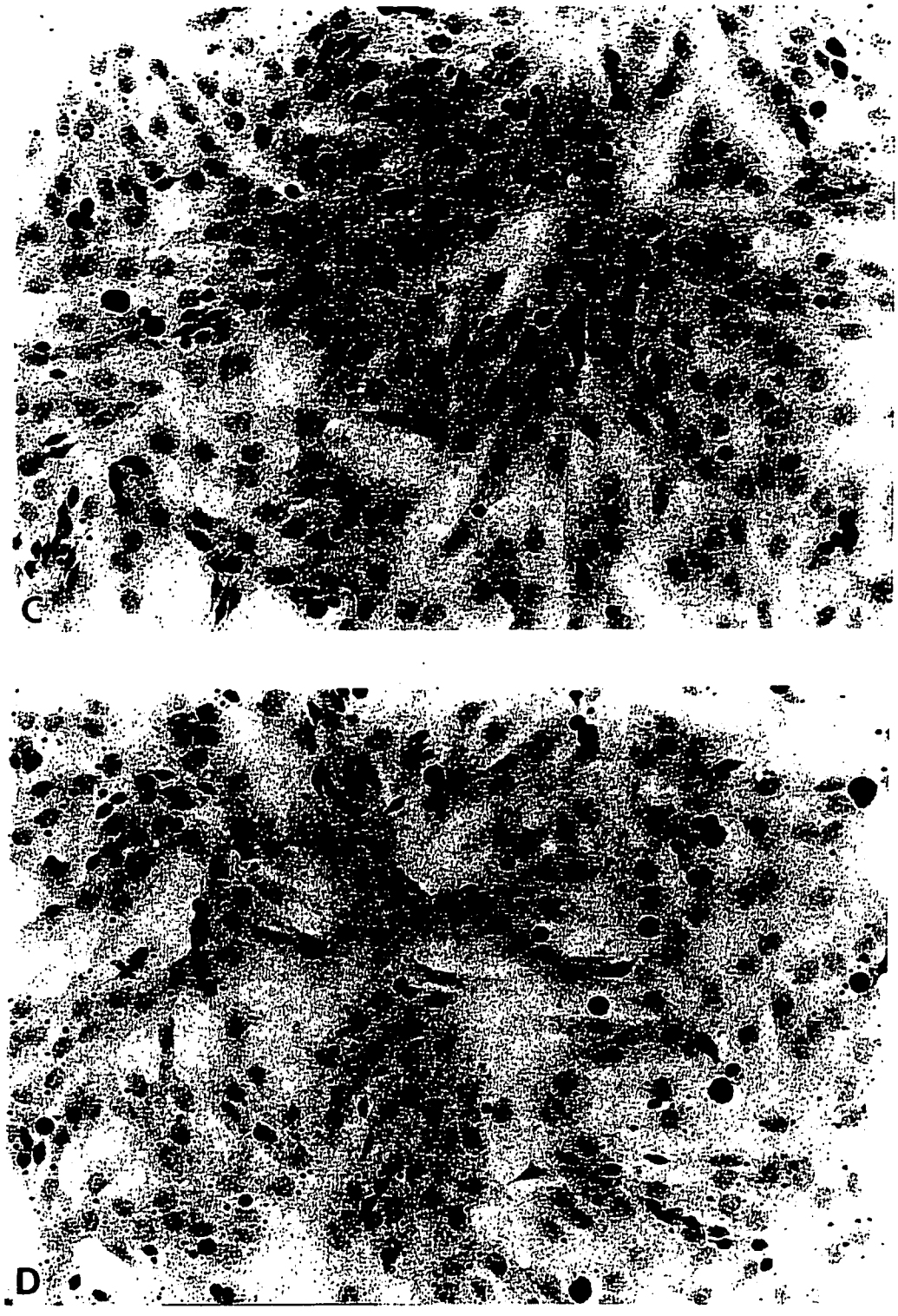

FIG. 1—*Continued.*

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-

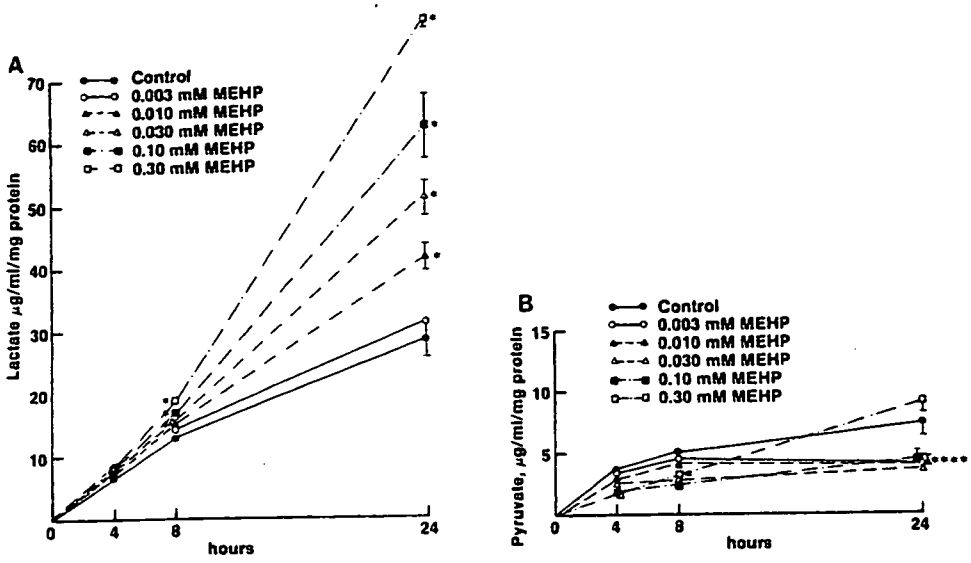

FIG. 2. (A and B) Concentrations of lactate and pyruvate in the medium during exposure to MEHP, mean ± SD, expressed as micrograms/milliliter/milligram cellular protein. Notice that while lactate increases with concentration and time, pyruvate is decreased by all but the highest concentration of MEHP. 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 µM cycloheximide was included. Cycloheximide decreased leucine incorporation 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 ± 4.9
0.003	32.6 ± 3.1
0.030	29.9 ± 4.7
0.30	31.0 ± 3.3
Medium	
0.0	13.3 ± .09
0.003	12.0 ± 2.7
0.010	13.4 ± 0.9
0.03	11.9 ± 1.5
0.10	10.5 ± 1.7
0.30	13.1 ± 0.3

[a] Values are expressed as means ± SD, dpm/µg 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 ± SD, moles/milligram cellular protein, n = 4.

* Significantly different from control.

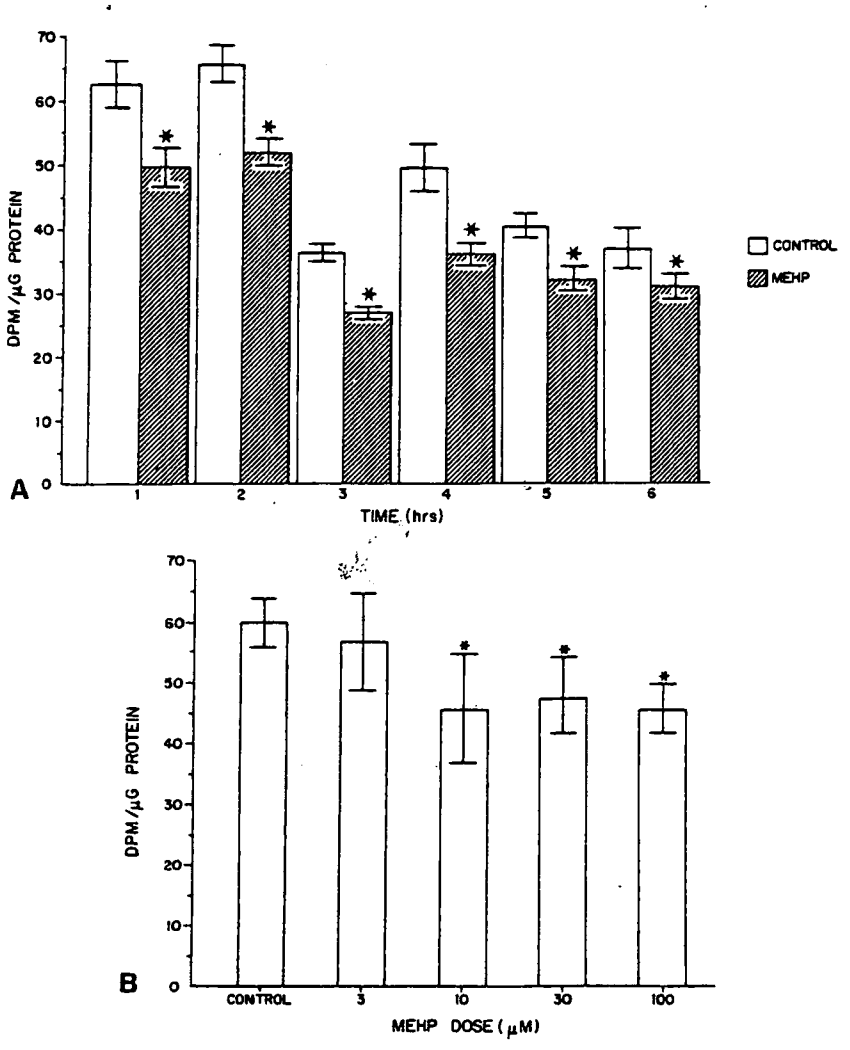

FIG. 3. (A) Time course of $^{14}CO_2$ production from [^{14}C]acetate during exposure to 0.1 mM MEHP. Values are expressed as the mean ± SD, dpms per minute per milligram cellular protein, n = 6. *Significantly different from control. (B) Generation of $^{14}CO_2$ from [^{14}C]acetate after 4 hr of 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 $^{14}CO_2$ production from [1,2-^{14}C]acetate. Exposure to 0.1 mM MEHP for as little as 1 hr decreased $^{14}CO_2$ 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}C]pyruvic acid was used as the substrate for $^{14}CO_2$ evolution, MEHP had no effect after 4 hr of treatment with MEHP (485 ± 84 dpm/hr/μg protein for controls; 520 ± 79 dpm/hr/μg protein for 100 μM MEHP; mean ± 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 MEPH 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 [^{14}C]acetate to $^{14}CO_2$. 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 [^{14}C]pyruvic acid $^{14}CO_2$ generation experiment. The lack of effect of MEHP on the production of $^{14}CO_2$ 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.

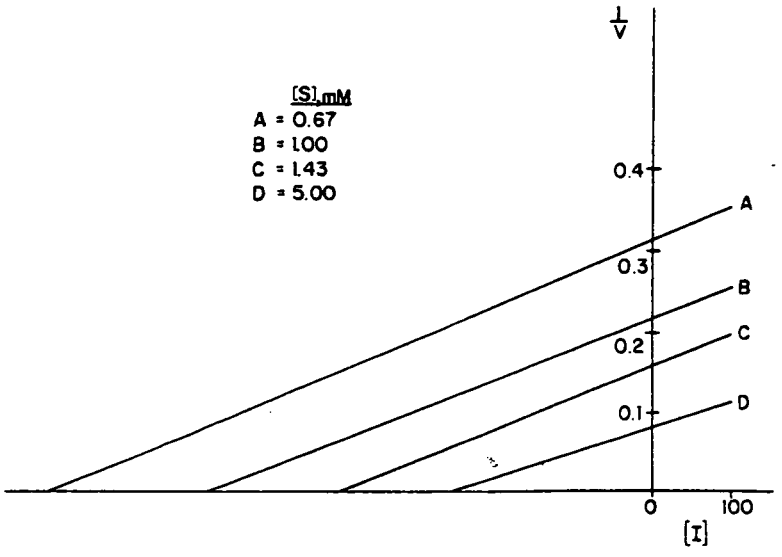

FIG. 4. Dixon plot ($^1/V$ vs [I] at different [S]) of the inhibition of SDH activity by MEHP. The slopes are not significantly different ($p > 0.1$).

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 $^{14}CO_2$-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 DMSO-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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The acute effects of mono(2-ethylhexyl)phthalate (MEHP) on testes of prepubertal Wistar rats

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Abstract

A single oral dose of 400 mg/kg body weight of mono(2-ethylhexyl)phthalate (MEHP), the testis toxic metabolite of di(2-ethylhexyl)phthalate, was given to 28-day-old male Wistar rats and the testis toxic effects were investigated 3, 6, and 12 h after exposure. Detachment and sloughing of germ cells were observed, and in the Sertoli cells the cytoplasmatic intermediate filament vimentin collapsed. In the immunohistochemical investigation the androgen receptor distribution was unchanged between the control group and treated groups. The expression of the testosterone-repressed-prostatic-message-2 gene in rat testis increased after 3 h, but returned to control levels after 6 and 12 h. Caspase-3 activity increased 3 and 12 h after MEHP exposure. This increase could not be correlated to an increase in DNA fragmentation or increase in apoptotic numbers of germ cells. In conclusion, the effect of MEHP in testis is apparently not involving the androgen receptor. Vimentin localisation in the Sertoli cells, and increased levels of caspase-3 activity appear to be sensitive and early markers of MEHP testis toxicity.

Keywords: DEHP metabolism; Vimentin; Caspase-3; TUNEL-staining; DNA laddering; *TRPM*-2 gene expression

1. Introduction

Di(2-ethylhexyl)phthalate (DEHP) and other phthalates are found widespread in food and the environment due to their use as plasticizers in consumer products, food packaging materials, and biomedical devices. A recent investigation by Blount et al. (2000) has demonstrated that the urinary levels of phthalates in humans are much higher than generally expected. Several studies in laboratory animals demonstrate the potential reproductive hazard of human exposure.

Young rats are more sensitive to DEHP than adults, since much lower doses (3.0–37 mg/kg bw per day) are able to induce histopathological effects in the testes of young rats compared to adult rats (1156 mg/kg bw per day) (Agarwal et al., 1986; Poon et al., 1997; Arcadi et al., 1998). In rats DEHP is rapidly hydrolysed in the gut to the major metabolite mono-(2-ethylhexyl)phthalate (MEHP) (Albro, 1986).

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