TABLE II

EFFECT OF PHTHALATE ESTERS ON SUCCINATE CYTOCHROME *c* REDUCTASE ACTIVITY OF LYSED MITOCHONDRIA[a]

Condition	Succinate cytochrome *c* reductase activity (nmoles × min^{-1} × mg $protein^{-1}$)
Control (0.24% ethanol)	118
MBP, 0.1 mM	125
MBP, 1.0 mM	112
DBP, 0.1 mM	76.7
DBP, 1.0 mM	66.1
MEHP, 0.1 mM	79.1
MEHP, 1.0 mM	27.1
DEHP, 0.1 mM	113
DEHP, 1.0 mM	116

[a]Conditions of the assay are described in Materials and Methods. All measurements were made in duplicate on the same mitochondrial preparation, and the mean values are presented. The results in this table are representative of 3 different preparations.

as the potentially rate limiting step. DBP and MEHP inhibited succinate cytochrome *c* reductase activity in the osmotically lysed mitochondria, while MBP and DEHP had no apparent effect (Table II). In each case that inhibition by DBP or MEHP was observed, the rate of reduction of cytochrome *c* was increased maximally by addition of NADH. Since NADH cytochrome *c* reductase and succinate cytochrome *c* reductase activities include the cytochrome b—c_1 region of the electron transport chain, then the inhibition by DBP and MEHP shown in Table II can be attributed to inhibition of SDH activity.

To characterize the nature of the inhibition by MEHP, mitochondrial SDH activity was determined as a function of succinate concentration at various MEHP concentrations. The data, presented in Fig. 2 as a Lineweaver-Burk plot, was analyzed by linear regression techniques to determine if the 3 regression lines were parallel. The slopes of these lines, which increased with increasing concentrations of MEHP, were significantly different ($P < 0.05$). Inhibition of mitochondrial SDH activity is concluded to be non-competitive, with an apparent $K_i = 2.4 \times 10^{-4}$ M. A characterization of DBP inhibition was not performed because of the difficulty in estimating the free concentration of this chemical which partitions rapidly into hydrophobic environments.

Energy-independent swelling induced by phthalate esters

The possibility that phthalate esters disrupt mitochondrial membrane integrity would also explain the loss of energy coupling in the oxidative phosphorylation and K^+ transport studies. Since such an effect would produce changes in OD independent of an energy source, the following studies were conducted in the absence of added respiratory substrates or

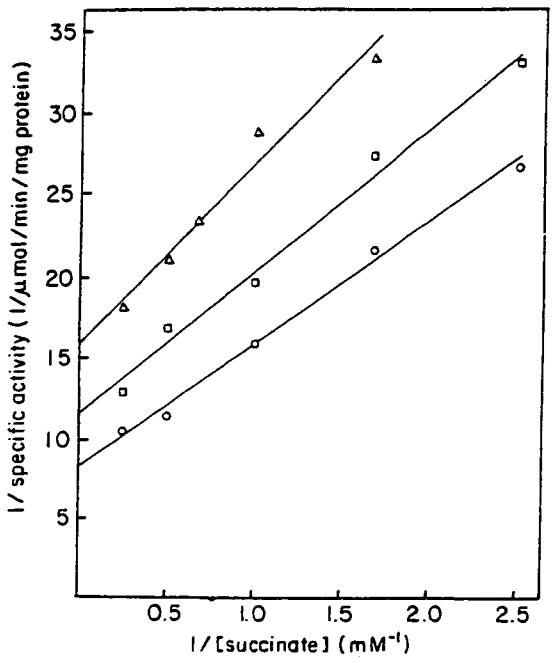

Fig. 2. Double reciprocal plots of SDH activity versus succinate concentration. SDH activity was measured as described in Materials and Methods. Straight lines of best fit were drawn after a least-squares linear regression of the data was performed. ○, control, slope = 3.48; □, MEHP = 6.7×10^{-5}M, slope = 4.06; △, MEHP = 1.7×10^{-4}M, slope = 5.08.

ATP. OD changes of mitochondria suspended under control conditions or with the various phthalate esters were compared (Fig. 3). In addition, suspensions of mitochondria were separately incubated for 5 min under the same conditions used to evaluate phthalate ester induced energy-independent swelling of rat liver mitochondria. After this incubation, supernatant and sediment fractions were separated by centrifugation (10 min at 8000 *g*), and malate dehydrogenase (MDH) activities or protein concentrations of these fractions were determined (Table III).

Incubation of mitochondria in the sucrose-KCl-Tris medium (Tracing *c*, Fig. 3) elicited no apparent change in OD, but there was a slight loss of MDH activity and protein into the supernatant fraction (Table III). Swelling of mitochondria in the control medium was not enhanced by the addition of valinomycin (results not shown); this finding was expected since the incubation did not contain an energy source. The addition of DEHP to the medium had no effect on the OD of the suspension (tracing *e*, Fig. 3) or on loss of MDH into the supernatant fraction (Table III) in comparison to the control condition. MEHP (tracing *f*) and DBP (tracing *b*) caused slight decreases in the OD of the mitochondrial suspensions (0.13 and 0.41 after 5 min, respectively) and correspondingly similar losses (~12%) of MDH activity or protein into the supernatant fraction (Table III). MBP (1.0 mM) caused the greatest OD change (tracing *g*) and led to the greatest loss of protein of the phthalate esters tested. The effect of 1.0 mM MBP on the loss of MDH activity into the supernatant fraction is not included since the

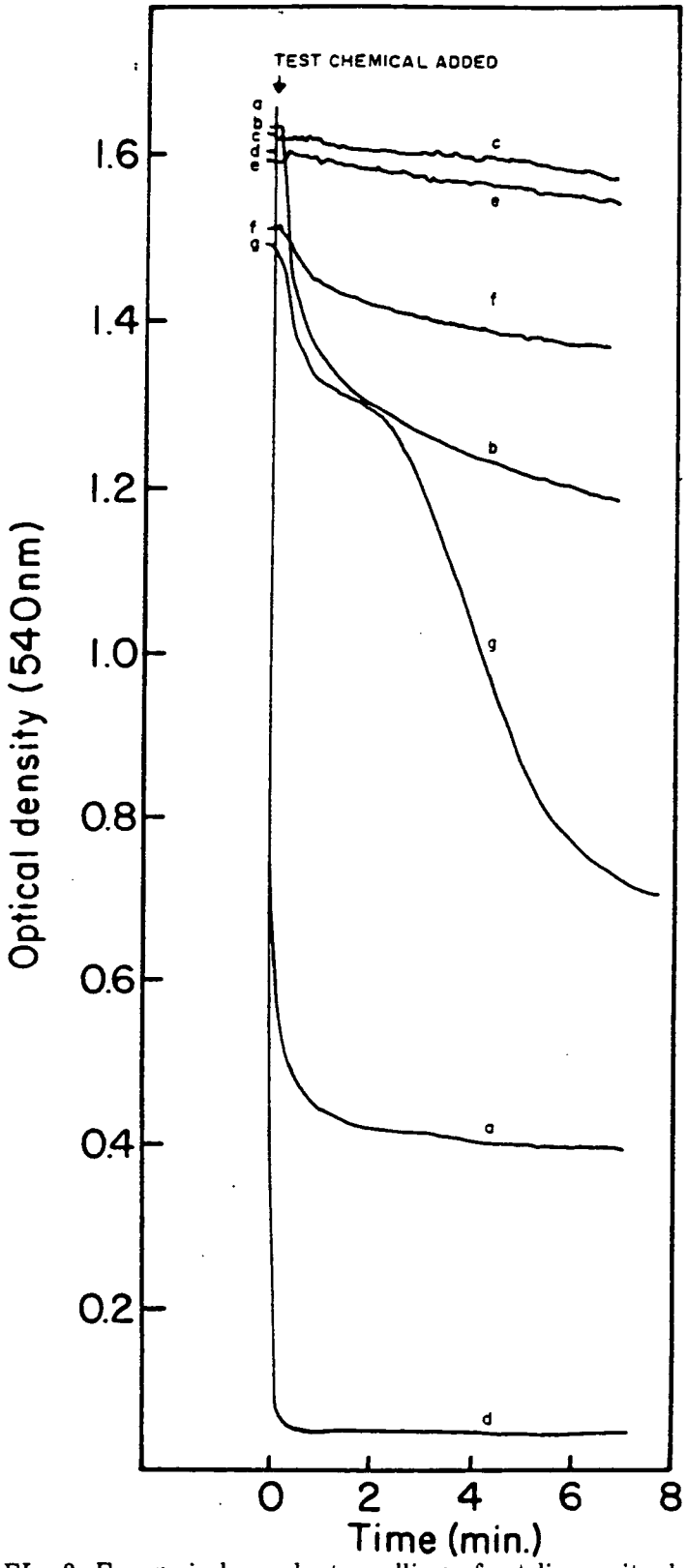

FIg. 3. Energy-independent swelling of rat liver mitochondria. Except for the tracing of mitochondria in distilled water, OD changes of isolated mitochondria (2 mg protein) were measured at 30°C in 3 ml of a medium that consisted of 250 mM sucrose, 25 mM KCl, 5 mM Tris—HCl (pH 7.6), plus the indicated test substance. a = distilled water; b = 0.1 mM DBP; c = control (0.24% ethanol); d = 0.05% Triton X-100; e = 1.0 mM DEHP; f = 1.0 mM MEHP; g = 1.0 mM MBP.

TABLE III

LOSS OF MALATE DEHYDROGENASE AND PROTEIN FROM RAT LIVER MITOCHONDRIA INCUBATED WITH PHTHALATE ESTERS

Condition[a]	Malate dehydrogenase (% activity in supernatant)	Protein (% in supernatant)
Control (0.24% ethanol)	4.5	7.5
1.0 mM MBP	—	23.5
0.1 mM DBP	12.3	11.2
1.0 mM MEHP	12.5	—
1.0 mM DEHP	4.2	—
Distilled water	46.5	38.0
0.05% Triton X-100	>99	—

[a]Mitochondria were treated as described in Fig. 3. After 5 min, samples were centrifuged for 10 min at 8000 *g*. MDH activities or protein concentrations of supernatant and sediment fractions were determined as described in Materials and Methods. All measurements were made in duplicate on the same mitochondrial preparation, and the mean values are presented. The results in this table are representative of 3 different preparations.

recovery of this activity was generally less than 50%. There was no difference in OD change between the tracings with 0.1 mM MBP (results not shown) and the control (tracing *c*).

The OD changes with the phthalate esters were much slower and less extensive than those resulting from treatment with 0.05% Triton X-100 (tracing *d*) or suspending the mitochondria in distilled water (tracing *a*). These studies indicate that uncoupling by MEHP and DBP is not due to drastic disruption of the inner mitochondrial membrane.

Tracing *g* of Fig. 3 indicates that greater uncoupling with 1.0 mM MBP may occur after a longer incubation with MBP, and that part of the OD changes observed during active K^+ uptake (Fig. 1) may be attributed to energy-independent swelling. Mitochondrial succinate respiration rates and energy-dependent K^+ uptake studies were therefore repeated after incubation for 7 min with 1.0 mM DBP. The longer incubation did not increase the effect of MBP on these energy-dependent processes.

DISCUSSION

The present studies demonstrate that phthalate esters uncouple mitochondrial energy-linked reactions, and inhibit (non-competitively) SDH activity. Inouye et al. [13] previously suggested that DBP acts as an uncoupler of oxidative phosphorylation, while Ohyama [12] and Takahashi [11] concluded that phthalate esters act mainly as electron and energy transfer inhibitors. Our studies on the effect of phthalate esters on energy dependent K^+-valinomycin induced swelling provide a means of distinguishing an uncoupler from an energy transfer inhibitor. This energy dependent process, whether driven by hydrolysis of ATP or oxidation of respiratory chain substrates, is sensitive to protonophore uncouplers; however, only the

ATP driven reactions are sensitive to energy transfer inhibitors, such as oligomycin. The evaluation of K^+ induced swelling driven by the oxidation of respiratory chain substrates included both succinate and ascorbate + TMPD as energy sources because of the specific inhibitory effect of phthalate esters on succinate oxidation. Since the effects of the various phthalate esters on energy dependent K^+ uptake were generally similar with ATP as with ascorbate + TMPD, it is concluded that the phthalate esters do not act as energy transfer inhibitors.

The effect of DBP on succinate respiration is concentration dependent; at low concentrations of DBP ($\leq$ 0.1 mM), the main effect is stimulation of state 4 respiration, while at higher concentrations (1.0 mM), DBP stimulates state 4 respiration and inhibits the succinate state 3 respiration rate. Since energy-independent swelling of mitochondria in the presence of DBP is associated with only slight release of malate dehydrogenase activity (12%), then stimulation of state 4 respiration by DPB is probably not due to loss of integrity of the inner mitochondrial membrane. It is suggested that stimulation of state 4 respiration is due to partitioning of DBP in the mitochondrial membrane causing a perturbation of the lipid phase that renders the membrane permeable to H^+ and other small ions. Inhibition of succinate state 3 respiration is attributed to inhibition of SDH activity, since succinate cytochrome *c* reductase activity was reduced by DBP and the inhibition of cytochrome *c* reduction was reversed by NADH.

In this study and that of Takahashi [11], DEHP had no apparent effect on respiratory control, or state 3 or state 4 succinate respiration rates; however, we did observe a slight impairment in ATP-driven K^+-valinomycin linked swelling with 1.0 mM DEHP. Inouye et al. [13] noted that state 3 respiration was reduced 18% by 1.0 mM DEHP. These effects may be due to inhibition of the adenine nucleotide translocator by DEHP [22], since ATPase activity of sonically prepared submitochondrial particles [15] was unaffected by DEHP or the other phthalate esters included in these studies (results not shown). Strivastava et al. [10] reported that DEHP inhibited mitochondrial SDH activity in vitro, while Takahashi [11] saw no effect of DEHP on either state 4 or state 3 succinate respiration rates of isolated rat liver mitochondria. In the present studies, DEHP had no effect on succinate respiration rates (Table I), succinate cytochrome *c* reductase activity (Table II) or succinate-dependent K^+-valinomycin induced swelling (Fig. 1). There is no readily available explanation for the apparent differences between the results of these studies and those of Strivastava et al. [10] on the effect of DEHP on succinate oxidation, however, contamination of DEHP with MEHP would result in inhibition of SDH activity.

Takahashi [11] reported that respiratory control ratios of isolated mitochondria in the presence of monoalkyl phthalates decreased as the alkyl chain length increased up to 7 carbons. The decrease in respiratory control with MBP was due to stimulation of state 4 respiration and inhibition of state 3 respiration [11]. In the present studies, a similar elevation in state 4 respiration was observed (Table I), but there was no decrease in succinate

state 3 respiration. In addition, we did not see inhibition of succinate cytochrome *c* reductase activity (Table II) or ATPase activity (results not shown) with MBP. Therefore, there is no apparent explanation for the reduced state 3 respiration rates reported by Takahashi [11]. Uncoupling caused by MBP was also apparent in the energy-dependent K^+-valinomycin linked swelling traces (Fig. 1). The effects of MBP on succinate, ascorbate + TMPD, or ATP-dependent K^+ uptake resemble those of an uncoupler at low concentrations [23]. Based on these results we suggest that MBP acts as a weak uncoupler. The energy-independent swelling traces (Fig. 3) show that 1.0 mM MBP induces swelling in isolated mitochondria which is accompanied by about 24% loss of protein (Table III). This suggests that uncoupling attributed to MBP may be partly due to a loss in integrity of the inner mitochondrial membrane. A contribution by proton shuttling across the inner membrane via the carboxyl group of MBP may also be a factor.

MEHP-induced uncoupling was evident by the stimulation of state 4 respiration (Table I) and impairment of energy-linked K^+ uptake with 3 different energy sources (Fig. 1). Loss of ATP-dependent K^+-valinomycin induced swelling was not due to inhibition of ATPase activity, since this enzyme was insensitive to MEHP (results not shown). Since MEHP inhibits SDH activity (Fig. 2) and uncouples oxidative phosphorylation, then 2 opposing effects on respiratory rates occur during succinate oxidation in coupled mitochondria. Stimulation of state 4 respiration by 0.1 mM MEHP is probably limited by the inhibitory effect on SDH activity. Inhibition of SDH activity could also account for the lower state 3 rate with 0.1 mM MEHP and the low state 3 and state 4 respiration rates with 1.0 mM MEHP. This explanation for the effect of MEHP on succinate respiration rates is supported by the finding that FCCP did not stimulate MEHP inhibited respiration, but addition of ascorbate + TMPD led to maximal respiratory activity. Energy-independent swelling was minimal in the presence of MEHP (Fig. 3), and MEHP caused only a slight loss of MDH activity (Table III). These results suggest that uncoupling induced by MEHP cannot be accounted for by loss of inner mitochondrial membrane integrity. Uncoupling by MEHP may occur as a result of an increase in membrane permeability to H^+ and other small ions due to an alteration in the molecular organization of the inner mitochondrial membrane components. MEHP does not act solely as a protonophore since it did not induce mitochondrial swelling in a medium containing potassium acetate plus valinomycin, and lacking an energy source (results not shown). Furthermore, MEHP probably does not act like the anesthetics chloroform and haloform which uncouple oxidative phosphorylation in the millimolar concentration range (1—2 mM), and which were suggested by Rottenberg [24] to act as energy transfer inhibitors between the proton electrochemical potential gradient and the ATPase complex.

The present studies show that phthalate esters affect isolated rat liver mitochondria by uncoupling energy-dependent processes and by inhibiting SDH activity. It is possible that permeability properties of other cellular

membranes are similarly altered by phthalate esters. The net effect of energy uncoupling and inhibition of substrate oxidation is a diminished chemical energy supply and altered regulation of ion transport in cells exposed to such compounds. These cellular changes could modify energy dependent cytoplasmic activities and the activities of ion-dependent enzymes. Since MEHP is a hydrolysis product of DEHP, it may play an important role in hepatotoxic effects associated with exposure to DEHP and in the growth inhibition by DEHP of human diploid fibroblasts in culture [4,5].

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Nephrotoxic effects of di-(2-ethylhexyl)-phthalate (DEHP) hydrolysis products on cultured kidney epithelial cells

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1 **Di-(2-ethylhexyl)-phthalate (DEHP) possesses a great industrial value as a plasticizing agent and has become an ubiquitous environmental contaminant. In most species it is rapidly metabolized to mono-(2-ethylhexyl)-phthalate (MEHP) and 2-ethylhexanoic acid (2-EHA). Evaluation of toxicity of DEHP and its primary metabolites has been focussed on reproductive toxicity and hepatocarcinogenic properties. The aim of this study was to determine the nephrotoxic potential of both DEHP metabolites by use of cultured kidney epithelial cells (Opossum kidney cells; OK cells).**

2 **For this purpose, OK cells were exposed for 3 days to MEHP and 2-EHA at concentrations ranging from 0.1 – 500 μmol/L and the toxicity as well as the effects on migratory activity and intracellular cytoskeleton were studied by cell biological, morphological and morphometric methods.**

3 **When compared with corresponding controls, treatment of OK cells with MEHP and 2-EHA, respectively, showed marked differences in cell viability between both DEHP metabolites. MEHP caused a dose-dependent decrease in cell viability (ED_{50} = 25 μmol/L) accompanied by a moderate swelling of the cells at concentrations up to 25 μmol/L. MEHP concentrations higher than 25 μmol/L caused a dose-dependent shrinkage of the cells and the occurrence of a high amount of cell debris as a result of cell lysis. 2-EHA did not cause a reduced viability or an altered cell volume. The migratory activity of OK cells was not significantly influenced by both metabolites. Moreover, MEHP toxicity resulted in a largely reduced and altered organization of F-actin (stress fibers), but not of myosin, microtubules and vimentin.**

4 **The study indicates that cultured epithelial cells can be used as a prescreening system to assess the nephrotoxicity of hazardous substances such as DEHP. As demonstrated in this study, only MEHP, but not 2-EHA, has a marked nephrotoxic effect *in vitro*.**

Keywords: di-(2-ethylhexyl)-phthalate; mono-(2-ethylhexyl)-phthalate; 2-ethylhexanoic acid; kidney; toxicity; cell culture

Introduction

Di-(2-ethylhexyl)-phthalate (DEHP) is a phthalic acid ester which is widely used as a plasticizing agent in polyvinyl chloride products and in medical devices. It is mixed to the plastic matrix to improve the flexibility of the material. But since DEHP is not chemically bound to the plastic polymer, it vaporizes quite easily. Due to the enormous amount of DEHP produced and the emissions during production, use and after disposal, DEHP has become an ubiquitous environmental contaminant and has been detected in water, soil and food.[1] As a consequence, it is incorporated in the human body and can be detected in the blood. Its use in blood storage bags and medical tubings has also led to significant exposures in certain patient categories.[2-4]

In most species, including man, DEHP is rapidly hydrolized in the liver to mono-(2-ethylhexyl)-phthalate (MEHP) and 2-ethylhexanol. The latter metabolite is rapidly oxidized to 2-ethylhexanoic acid (2-EHA).[5] Thereafter, the metabolites undergo further biotransformation steps which vary with the species examined.[6] A number of studies have been carried out to estimate the potential human hazard of DEHP. The majority of these studies focussed on the reproductive toxicity[2,7] and on the hepatocarcinogenic properties.[8] Another sub-lethal chronic effect of DEHP is its influence on the liver by causing swelling and an increased weight.[9] At the cellular level an increased pigmentation, occurrence of fatty vacuoles and fatty degeneration can be distinguished after exposure to DEHP.[9,10] Most of the observed toxic effects have been reported not to be caused by DEHP itself, but by its further metabolites.[11,12]

It has been reported by Schmid and Schlatter[13] that after oral dosing of volunteers with DEHP, 11 – 15% of the dose was excerted in the urine, mainly in the form of conjugated metabolites, with MEHP being the most important compound. However, only little attention has been drawn to the

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nephrotixic effects of MEHP and 2-EHA as the metabolites of DEHP.

Prompted by this background, this study was undertaken to examine possible nephrotoxic effects of MEHP and 2-EHA in cultured kidney epithelial cells. For this purpose, cells were exposed to both metabolites at different concentrations and the toxicity as well as the effects on migratory activity and intracellular cytoskeleton were studied by cell biological, morphological and morphometric methods.

Materials and methods

DEHP hydrolysis products

2-EHA was obtained from Aldrich Chemicals (99% purity; Steinheim, Germany) and used without further purification. MEHP was synthesized by esterificiation of phthalic anhydride (Aldrich Chemicals, Steinheim, Germany) with 2-ethylhexanol (Aldrich Chemicals, Steinheim, Germany) in pyridine and purified by HPLC (RP-8, acetonitrile, water, acetic acid) to obtain a purity of at least 99.5% as determined by gas chromatography.

Cell culture

The experiments were performed on Opossum kidney epithelial cells (OK cells) of the proximal tubule. Cells were maintained in culture in a humidified atmosphere of 6% CO_2 and 94% air in Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 nutrient mixture (1+1; v/v) containing 50 IU/mL penicillin, 50 μg/mL streptomycin, 10% fetal calf serum and 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES). All cell culture reagents were purchased from Gibco–BRL, Eggenstein, Germany. Cells were routinely subcultured at confluency by trypsin/ethylene-diamintetraacetic acid (EDTA) treatment (0.05%/0.02%; pH 7.2). Cell shape was examined at phase contrast using an inverted microscope (Nikon, Düsseldorf, Germany).

Test assays

For the examination of toxicity, OK cells were seeded at a density of 2000 cells/cm² into 6-well plates (Costar 3506; Tecnomara, Fernwald, Germany) and were allowed to attach and spread for 24 h. Thereafter, culture medium was exchanged and fresh culture medium with 1% fetal calf serum to achieve cell quiescence and containing the appropriate concentration of MEHP and 2-EHA was added. The concentrations of both DEHP hydrolysis products ranged from 0.1–500 μmol/L. After an incubation period of 3 days, cells were washed twice with phosphate-buffered saline (PBS) to remove cell debris as well as detached and floating cells. Still adherent and viable cells were detached by trypsin/EDTA treatment for 10 min at 37°C, diluted with an isotonic solution (CASYton; Schärfe System, Reutlingen, Germany) and homogenous cell suspensions were achieved by vortexing for at least 1 min. An aliquot of the cell suspensions was used for measuring cell number and mean cell volume with a cell counter/analyzer system (Schärfe System, Reutlingen, Germany) as described in detail elsewhere.[14,15]

Cell migration was measured by seeding OK cells at a density of 10 000 cells/cm² into 6-well plates (Costar 3506; Tecnomara, Fernwald, Germany) and cultivating the cells for 3 days until confluency was reached. Then, the adherent cell layer was artificially 'wounded' by producing a cell-free area. For this purpose, a sterile razor blade was carefully put on the cell layer to achieve a sharp wound edge and the cells on one side of the blade were removed with a sterile cotton tip. After a wash with PBS, fresh culture medium with 1% fetal calf serum and containing the appropriate MEHP and 2-EHA concentrations was added. After 3 days, cells were fixed and stained with an azur B-eosin solution (Romanowsky-Giemsa staining; Serva Feinbiochemika, Heidelberg, Germany).

Visualization of cytoskeleton by epifluorescence microscopy

For the visualization of cytoskeletal alterations due to MEHP and 2-EHA treatment, 20 000 cells were seeded onto glass coverslips (17 mm in diameter) in 12-well plates (Costar 3512; Tecnomara, Fernwald, Germany) and were allowed to attach and spread for 24 h. The cells were subsequently incubated for 3 days with 50 μmol/L with MEHP and 2-EHA, respectively. Thereafter, cells were fixed and permeabilized as follows: (i) methanol for 6 min at −20°C for indirect immunofluorescence; (ii) 3.5% formaldehyde in PBS for 15 min at 25°C, 1% Triton X-100 in PBS for 2 min at 25°C for staining of F-actin. For indirect immunofluorescence microscopy the following first antibodies were used: (i) monoclonal anti-pan myosin (Amersham Buchler, Braunschweig, Germany); (ii) anti-monoclonal α-tubulin (Amersham Buchler, Braunschweig, Germany); (iii) monoclonal anti-vimentin (Camon, Wiesbaden, Germany). Texas red-conjugated second antibodies were purchased from Dianova (Hamburg, Germany). Controls for the specific staining were done by omitting the first specific antibody and applying only the second antibody. F-actin was stained by incubation of the fixed and permeabilized cells with 2 μg/mL tetramethylrhodamine isothiocyanate (TRITC)-phalloidin (Sigma Chemie, Deisenhofen, Germany) for 15 min at 25°C. Samples were mounted in Mowiol 4-88 (Hoechst, Frankfurt, Germany) and examined and photographed with a Nikon Optiphot microscope (Nikon, Düsseldorf, Germany) equipped for epifluorescence

with the appropriate filter sets and using a Nikon Planapo 40/1.0 oil immersion lens. Micrographs were taken on Kodak Tri-X pan 400 film.

Morphometric analysis
The fluorescence intensity of the stained samples was examined by epifluorescence with a microphotometer (Nikon, Düsseldorf, Germany) mounted at the Nikon optiphot microscope and using a Nikon Planapo 60/1.4 oil immersion lens. In three independent experiments a minimum of 10 arbitrarily selected visual fields was examined for each coverslip.

Statistical analysis
The data are given as arithmetic means ± standard error of the mean (s.e.m.). Statistical analysis was made by Student's *t*-test. Statistical significance was assumed at $P<0.05$.

Results

Effect of MEHP and 2-EHA on cell viability and cell volume
In comparison to the corresponding controls, treatment of OK cells with MEHP and 2-EHA at concentrations ranging from 0.1 – 500 μmol/L showed marked differences in cell viability between both DEHP hydrolysis products. The exposure of cells to MEHP resulted in a dose-dependent decrease of viability (Figures 1A and 2A). The ED_{50}, i.e. the effective dose causing a 50% loss in viability, was calculated from the dose-response curve to be 25 μmol/L for MEHP. At concentrations up to 25 μmol/L the decrease in viability was accompanied by a moderate cell swelling (Figures 1B and 2A). MEHP concentrations higher than 25 μmol/L caused a dose-dependent shrinkage of the cells and the occurrence of a high amount of cell debris as a result of cell lysis.

In contrast, 2-EHA did not cause a significantly reduced cell viability (Figures 1A and 2B) or alterations in cell volume (Figures 1B and 2B) when compared with corresponding controls. Therefore, an ED_{50} cannot be given for 2-EHA.

Effect of MEHP and 2-EHA on cell migration
Since cell migration can be used as one indicator of the wound healing process after epithelial cell damage, we additionally checked the effect of both DEHP metabolites on the migratory activity of OK cells. Although both hydrolysis products slightly enhanced the migratory activity of OK cells, no significant differences to the corresponding controls could be observed at all concentrations tested (Figure 3). The basal migration distance of the controls without metabolite treatment was measured to be 105.6 ± 11.9 μm/day (mean value ± s.e.m.; n=4).

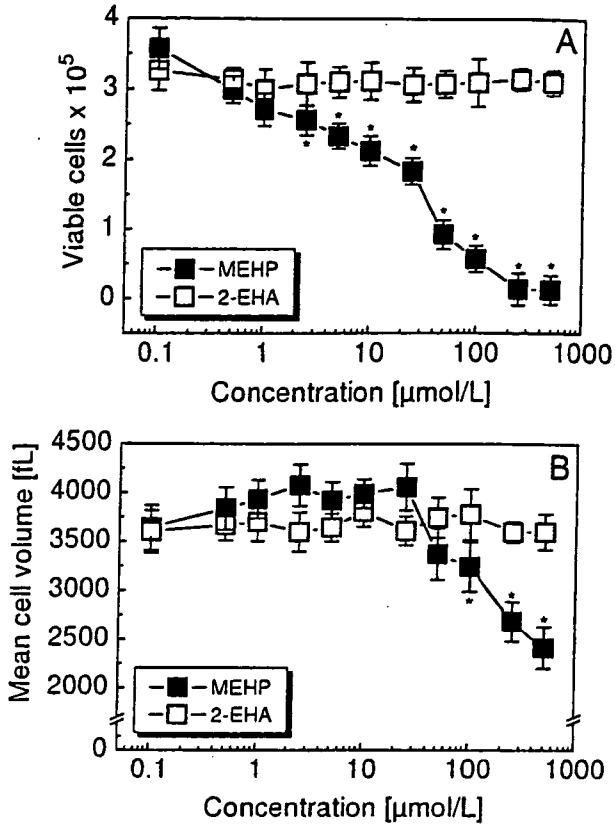

Figure 1 Effect of MEHP and 2-EHA on cell viability (A) and cell volume (B) of Opossum kidney cells after an incubation period of 3 days. Note that MEHP, but not 2-EHA, causes a dose-dependent loss of viability accompanied by a moderate swelling of the cells at concentrations up to 25 μmol/L and a shrinkage of the cells at concentrations higher than 25 μmol/L. Data represent mean value ± s.e.m. of three independent experiments with duplicate wells. $^*P<0.05$ (Student's *t*-test)

Effect of MEHP and 2-EHA on cytoskeletal organization
As previously described in detail,[16] F-actin was observed in OK cells to be distributed in two distinct domains (Figure 4A). At the base of the cells attached to the substrate (basolateral surface) F-actin was organized in the form of long bundles of stress fibers mainly at the cell margins with a thin meshwork across the whole cell. At the apical domain F-actin was not organized as stress fibers, but in the form of small and highly fluorescent spots distributed across the entire cell. The F-actin seen as spots on the cell surface represents actin arrayed in the core of many microvilli that cover the surface of epithelial cells.[17,18]

In the case of MEHP treatment, apically located fluorescent spots were largely reduced, whereas the basolateral stress fiber bundles were retracted and present only in single areas at the cell periphery (Figure 4B). 2-EHA treatment caused no alterations in stress fiber organization (not depicted). This loss

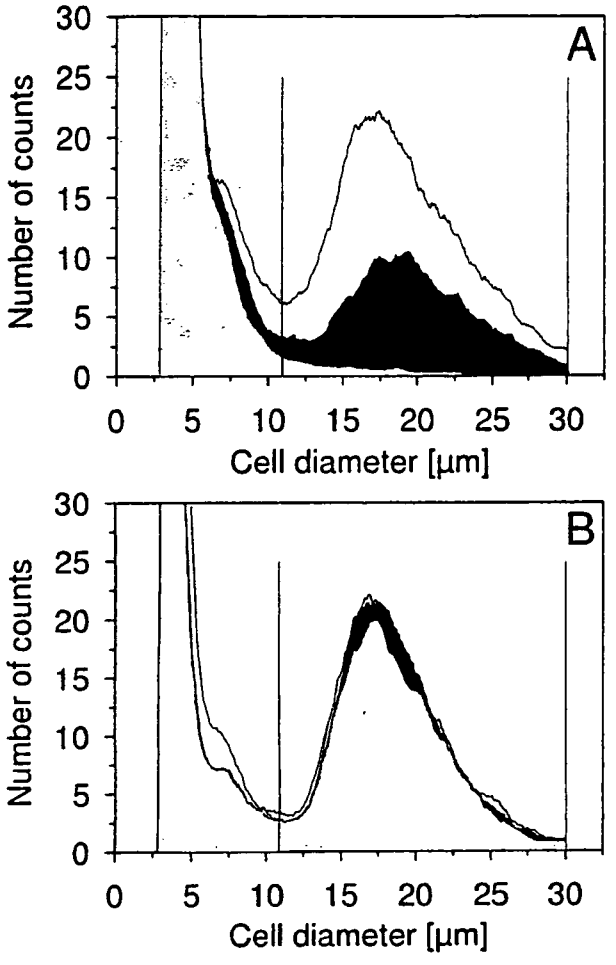

Figure 2 Original cell size plots of the counter/analyzer system representing the different toxicity of MEHP (A) and 2-EHA (B after 3 days. Open area = corresponding controls; black area = 25 µmol/L; hatched area = 250 µmol/L

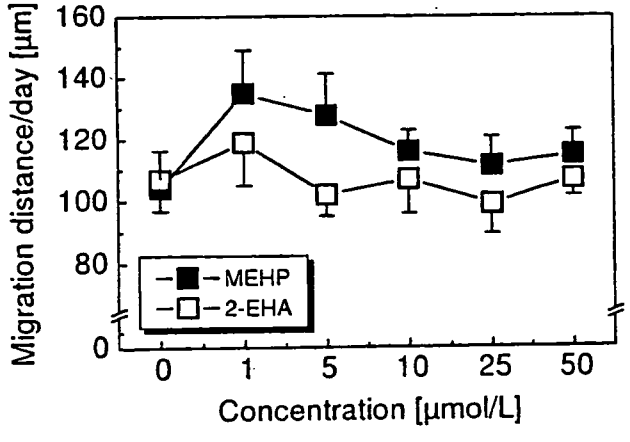

Figure 3 Effect of MEHP and 2-EHA on cell migration of Opossum kidney cells. Both DEHP hydrolysis products do not influence the migratory activity of the cells. Data points represent mean value ± s.e.m. of three independent experiments

in stress fiber network as caused by MEHP treatment was accompanied by a significant decrease in fluorescence intensity by 58.9 ± 11.9% (mean value ± s.e.m.; n=3; $P<0.01$; Student's t-test) when compared with corresponding controls. In contrast, 2-EHA treatment reduced the fluorescence intensity of intracellular stress fibers only slightly by 17.3 ± 10.9% (mean value ± s.e.m.; n=3).

MEHP and 2-EHA treatment did not cause a reorganization of myosin (Figure 4C and D), microtubules (Figure 4E and F) or vimentin (not depicted) as the main type of intermediate-sized filament present in OK cells. Myosin was distributed in a regular periodic fashion in form of aggregates throughout the whole cytoplasm of the cells. Microtubules initiated at microtubule organizaing centers (MTOCs) in the perinuclear region and ran through the cytoplasm to the cell periphery. Vimentin was distributed in the form of a prominent perinuclear ring with thin, wavy and branched filaments running to the cell periphery.

Discussion

In higher mammals, DEHP is metabolized very rapidly. After absorption the enterogastric system and the liver are mainly responsible for the metabolism of DEHP, whereas the kidney plays only a minor role for the breakdown. The most important metabolites are phthalic acid and MEHP and its derivatives which are excreted via the urine and bile.[19] Despite the rapid metabolism and excretion of DEHP, an accumulation in tissues and organs of mammals, birds and fish has been noted. Although phthalate esters such as DEHP are suggested to be of low acute toxicity,[20] studies in rodents and other mammals have shown that DEHP administered in large quantities may induce hepatocellular carcinomas,[21,22] gene mutations, teratogenic and fetotoxic effects[23] and reproductive disorders.[7,24] In addition, Crocker *et al.*[25] found an impaired renal function and renal cysts in rats given oral doses of DEHP over a period of 1 year.

Although a number of *in vitro* studies on the effects of DEHP have been published during the last years[7] to our knowledge, this study is currently the first one demonstrating the effects of DEHP hydrolysis products on cultured cells of the kidney. As clearly shown, only MEHP, but not 2-EHA, causes a dose-dependent decrease of cell viability. Previous results show that especially MEHP is more hazardous than DEHP as the parent compound.[7,24,26-28] Moreover, in the case of MEHP treatment a moderate swelling of the cells at lower concentrations was observed pointing to a necrotic process causing cell death.[29] The shrinkage at MEHP concentrations higher than 25 µmol/L is due to dead cells which ruptured and underwent a lysis process with cell fragments of a low volume. 2-EHA which is used for acid-based pesticide fomulations and has been designed to replace pentachlorophe-

nol-containing agents, did not affect cell viability and cell volume. 2-EHA has been evaluated for its biochemical safety, i.e. for its tumor-inducing potency, in the peroxisome-proliferator test[30] with equivocal results. In addition, 2-EHA was also shown to inhibit urea synthesis and to stimulate carnitine acetyltransferase activity in rat liver mitochondria.[31] As far as we know, 2-EHA has not been evaluated in a cytotoxic assay using kidney cells so that the results of this study can be taken as a first indication proving its minor nephrotoxicity.

Somewhat equivocal are the results of cell migration and cytoskeletal alterations as observed for MEHP. Although the migration distance is not significantly influenced after exposure to both DEHP hydrolysis products, the intracellular stress fiber network is largely altered in the case of MEHP. Moreover, the fluorescence intensity of the intra-

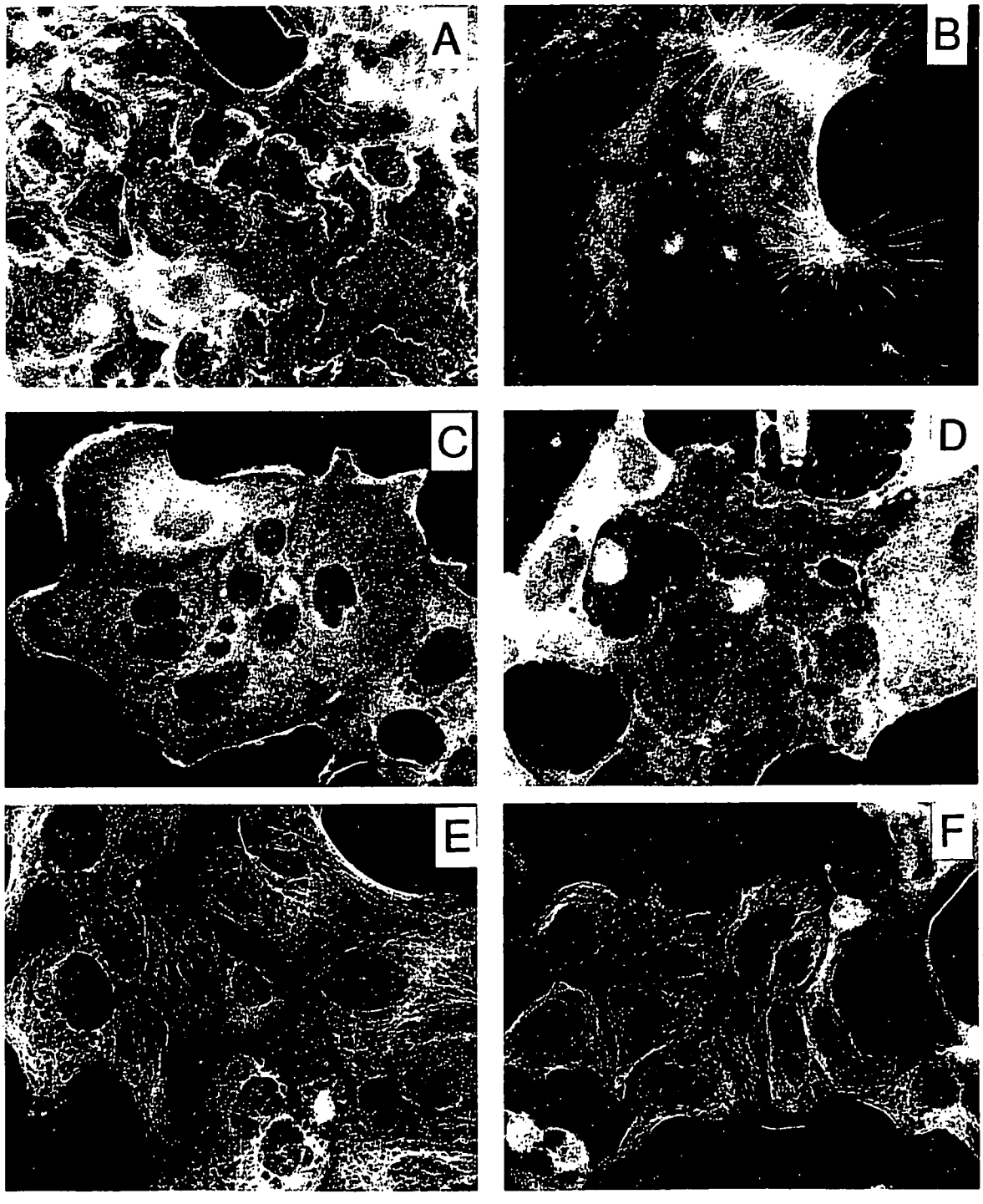

Figure 4 Effect of MEHP (**B,D,F**) in comparison to untreated cells (**A,C,E**) on the organization of intracellular stress fibers (**A,B**), myosin (**C,D**) and microtubules (**E,F**). While MEHP-treatment causes a marked loss of stress fibers and myosin aggregates, microtubules and vimentin (not depicted) remain unaltered. Cells treated with 2-EHA exhibit a cytoskeletal organization similar to untreated controls. Epifluorescence microscopy; magnification: × 600

cellular stress fibers which is a marker for its state of organization is also reduced after MEHP exposure. It is well known that cell movement requires a dynamic equilibrium between actin subunits (globular actin) and polymerized actin (filamentous actin) which is arranged in the form of stress fiber bundles in cultured cells.[18] When the stress fiber organization is altered by a depolymerization of filamentous actin to subunits as observed for MEHP, the migratory activity of the cells should also be reduced. The pattern of distribution of myosin, an actin-binding protein also present in stress fibers, was observed to be unaltered. One may speculate that MEHP primarily causes a loss in cell membrane integrity. Since stress fibers are anchored at the inner cell membrane via vinculin, the rearrangement of stress fibers might be the result. A more satisfactory explanation for this contradictory result cannot be given yet and needs further investigation.

In conclusion, the isolated toxicity of the unconjugated metabolites MEHP and 2-EHA on kidney cells as demonstrated in this study, may be difficult to assess, because the experimental results of our study may not be completely comparable to the situation in human primates.[32] As already well known, the initial step in metabolism of DEHP is a partial hydrolysis to MEHP, followed by oxidation of the side chain. The rat is the only species tested that excretes free metabolites exclusively.[6] In humans, unconjugated DEHP and MEHP are not excreted in the urine, but glucuronide conjugates of MEHP and related metabolites are excreted.[13,33]

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Short communication

THE BIOLOGICAL ACTIVITIES OF PHTHALATE ESTERS ON RAT GASTRIC MUSCLE

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Monoethylhexyl phthalate, at concentrations that can occur in blood stored in plastic bags (0.1-0.5 mg/ml), reduced contractions of rat isolated gastric fundus to PGE_2 and acetylcholine; the diethyl compound was less effective. In contrast, dibutyl phthalate (1 and 10 μg/ml) and, to a lesser extent di-isobutyl phthalate, increased the muscle tone. These results are discussed in relation to blood transfusion, and to structural similarities between phthalates and prostaglandins.

Phthalates Prostaglandins Blood transfusion Smooth muscle

1. Introduction

Phthalate esters are used extensively as plasticizers, and they form 30 to 40 per cent of various plastics. These enter human tissues (Jaeger and Rubin, 1970; Fishbein and Albro, 1972; Thomas et al., 1978; Thomas and Northup, 1982), and although they have low acute toxicity their chronic toxicity is not fully known (Rock et al., 1978). We have studied phthalate esters in relation to prostaglandins for three reasons: (1) they contributed to the biological activity extracted from blood stored in plastic bags (unpublished data); (2) diethylhexyl phthalate can cross-react with prostaglandin antibodies (Kindahl and Granström, 1980); and (3) butylidenephthalide, isolated from a rhizome, inhibited rat uterine contractions to $PGF_{2\alpha}$ (Ko, 1980).

2. Materials and methods

2.1. Phthalates

Di-isobutyl phthalate (DIBP) (Eastman Kodak Company, NY), di-n-butyl phthalate (DBP) and diethylhexyl phthalate (DEHP) (Aldrich Chemical Company Ltd.) were purchased commercially. Monoethylhexyl phthalate (MEHP), the main metabolite of the diethyl compound (DEHP), was synthesized by refluxing phthalic acid (4.2 g), 2-ethylhexan-1-ol (3.9 g) and concentrated sulphuric acid (0.1 ml) for 1 h. Following chloroform extraction and evaporation to dryness, the residue was dissolved in hexane and applied to a silicic acid column (50 g) which was eluted successively with 300 ml amounts of: hexane; hexane : ether 95 : 5 (v/v); hexane : ether 75 : 25; and ether, monitored spectrometrically at 220 nm. DEHP eluted with hexane : ether 95 : 5 while MEHP eluted with 75 : 25 fraction. MEHP chromatographed as a single peak on reverse phase HPLC monitored at 210 nm (Spherisorb 5 μm ODS Pye Unicam Ltd MeOH : H_2O : H_3PO_4 80 : 20 : 0.1, retention time 6.25 min). The structure of MEHP was confirmed by UV and mass spectrometric analysis; its full UV spectrum, recorded on a Carey spectrophotometer, showed a λ_{max} of 274 nm, with a shoulder at 280 nm, consistent with the presence of a phthalate. Mass spectra were obtained in the electron impact mode on a Kratos MS 50 (70 ev 150°C) and under fast atom bombardment (FAB)

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ionisation using VG Analytical Zero Alpha Beta-High Field analysis. In the FAB experiment, using xenon as the primary ionising beam, there was a intense quasimolecular ion ($M + H^+$) of M/z 279, which shifted to $(M + H)^+$ M/z 293 following esterification of MEHP with diazomethane. The electron impact spectra of the free acid (M/z 278, 249, 222, 221, 179, 163, 149) and the methyl ester (M/z 292, 261, 236, 235, 195, 181, 163, 149) further defined the covalent structure of MEHP.

2.2. Bioassay

The phthalate esters were dissolved in absolute ethanol or suspended finely in 150 mM NaCl and examined for their effects on rat gastric muscle. Strips of gastric fundus were suspended under a load of 0.5 g in Krebs solution (NaCl 118.5, $CaCl_2$ 2.5, KH_2PO_4 1.2, KCl 4.7, $MgSO_4$ 1.2, $NaHCO_3$ 2.5, dextrose 11.7 mM) at 37°C and bubbled with 5% CO_2 in O_2. The muscle tone and responses of each strip were recorded using an isotonic transducer and pen recorder. Effects on muscle tone were determined after obtaining consistent submaximal control contractions to PGE_2 and after examining the effect of the ethanol vehicle controls (10 μl, 5 min contact time). Phthalates 1-100 μg/ml in 10 μl ethanol were added to the bathing solution (5 min contact) followed by further washouts at 5 min intervals if needed to return the tissue tone to normal.

The phthalates in ethanol were also studied using tissues bathed in Krebs solution containing hyoscine, mepyramine, methysergide, phenoxybenzamine, pronethalol and indomethacin (respectively 0.2, 0.2, 0.1, 0.1, 1 and 1 μg/ml) to

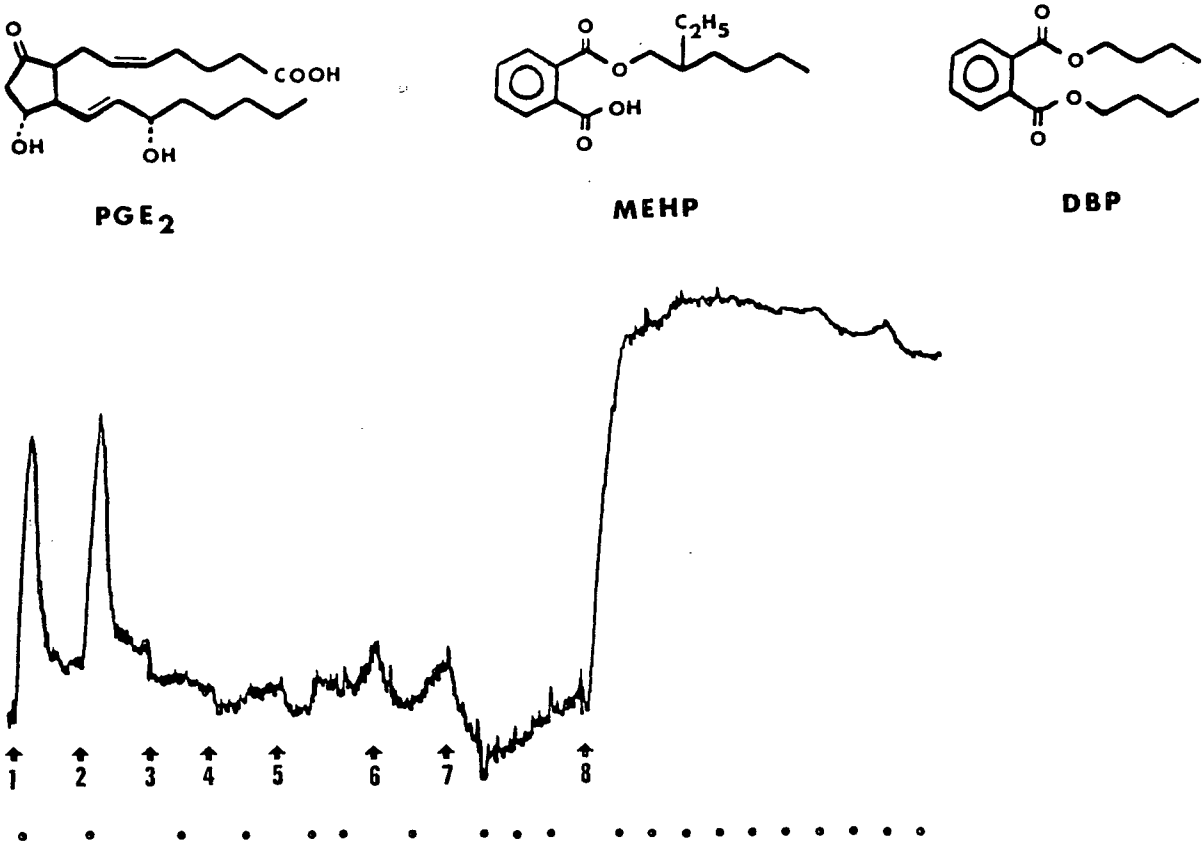

Fig. 1. The effect of phthalates on the tone of rat gastric fundus in the absence of amine-blocking drugs and indomethacin. The numbered arrows represent final bath concentrations of: PGE_2 1 ng/ml (arrows 1 and 2; 10 min between additions, 90 s contact time); ethanol vehicle 2 μl/ml (3 and 4; 5 min contact); MEHP 1, 10 and 100 μg/ml respectively (5, 6 and 7; 5 min contact); DBP 100 μg/ml (5 min contact). Each dot represents washout of the tissue bath. MEHP caused a small concentration-related drop in muscle tone, whereas DBP causes a marked long-lasting increase in tone which persisted despite many changes in bath fluid.

block receptors for acetylcholine (ACh), histamine, 5-HT, α- and β-adrenoceptor agonists, and to inhibit prostaglandin synthesis (Bennett et al., 1973). Consistent submaximal control contractions to PGE_2 (contact time 90 s) were obtained using a 10 min cycle time. Phthalate solution or vehicle control was then added immediately after washing out each dose of agonist, and the phthalate dose was increased if it produced no agonist effect or no antagonism of PGE_2.

In 6 other experiments DEHP and MEHP suspended in 150 mM NaCl solution were tested similarly in the presence of all the antagonists except hyoscine against fundus contractions to alternate submaximally effective doses of ACh and PGE_2. Matched vehicle controls were studied on a separate strip from the same gastric fundus. The vehicle usually had little or no effect, but in order to control for this and for spontaneous changes in sensitivity to PGE_2 or ACh any percent change in the response of the control strip was subtracted from that in the test strip.

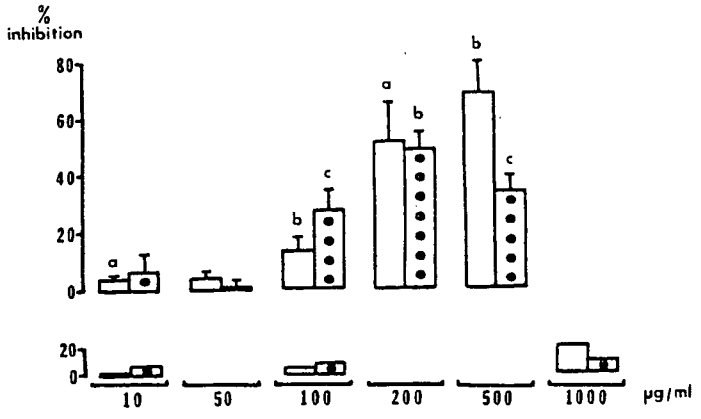

Fig. 2. The effect of MEHP (top) and DEHP (bottom) on alternate submaximal contractions to PGE_2 (open histograms) and acetylcholine (dotted histograms) by rat gastric fundus in the presence of indomethacin and amine-blocking drugs except hyoscine. These drugs greatly reduced the tissue tone but MEHP and DEHP had no further effect. At higher concentrations (100-500 μg/ml) MEHP caused a long-lasting inhibition of contractions to PGE_2 and acetylcholine which disappeared only gradually after several washouts. DEHP 1 mg/ml showed a little inhibitory activity against PGE_2 and acetylcholine. The vehicle alone had little or no effect in simultaneous control experiments. n = 6; P values (paired t-test) $^a < 0.05$; $^b < 0.02$; $^c < 0.005$.

3. Results

In the absence of the mixture of blocking drugs MEHP 1-100 μg/ml final bath concentration caused a small concentration-related relaxation. The tissue tone returned to normal 1-18 min after changing the bath fluid, this return taking longest with the highest concentration (fig. 1). In contrast, DBP 1-100 μg/ml caused a concentration-related contraction which subsided very slowly after washout (fig. 1) with little recovery over the 3 h after washing out the highest concentration. DIBP, DEHP or vehicle controls had little or no effect (3 experiments).

The mixture of blocking drugs, used in another 3 experiments, markedly reduced the tissue tone and spontaneous activity, thus tending to mask inhibitory responses and to increase the detection of excitatory responses. DBP 1-10 μg/ml caused a marked increase in tone which was long-lasting and required several changes of bath fluid before the tissue returned to its original baseline. Muscle tone increased slightly with DIBP 10 μg/ml and returned to normal after washout. DEHP (1-500 μg/ml) did not affect the tissue tone or the PGE_2 responses, whereas MEHP (1-500 μg/ml) caused a concentration-related inhibition of PGE_2 responses without altering the tissue tone.

In further experiments using all the blocking drugs except hyoscine, DEHP produced only a small inhibition of contractions to PGE_2 or ACh, but its metabolic product MEHP 1-500 μg/ml reduced contractions to PGE_2 or ACh by 4-69% and 1-48% respectively (n = 6, fig. 2). MEHP 200 μg/ml, a concentration within the range that can occur with plasma stored in plastic bags, reduced contractions to PGE_2 or ACh by $51.1 \pm 13.9\%$ and $47.7 \pm 6.7\%$ respectively ($P < 0.05$ and $P < 0.02$). The inhibition occurred within ~ 8 min contact of MEHP with the tissue (the time between washing out the control dose of PGE_2 or ACh and administering the next dose in the presence of MEHP); it was long-lasting and several washouts were needed to return the responses to normal.

4. Discussion

Dibutyl phthalate, which has some structural resemblance to prostaglandins (fig. 1), was mod-

erately potent at increasing the tone of rat gastric fundus. The di-iso-isomer acted similarly but was less potent. These phthalates may act as prostaglandin mimetics, a possibility strengthened by the presence of the amine-blocking drugs in the bathing fluid, and by the interaction of DEHP with prostaglandin antibody (Kindahl and Granström, 1980).

Diethylhexyl phthalate (DEHP) is the most widely used plasticizer, and is therefore to be found almost everywhere in the Western world. DEHP had little activity on rat fundus, whereas 100-500 μg/ml of its principal metabolite MEHP (fig. 2) antagonised responses to PGE_2 and ACh. It might be worth examining phthalate analogues as prostaglandin agonists and antagonists. Transfusion blood stored in polyvinyl chloride plastic bags extracts DEHP at a daily rate of 0.25-0.03 mg/100 ml (Jaeger and Rubin, 1972), and metabolises it to MEHP (Rock et al., 1978). Transport and storage conditions of plasma and 25% normal serum albumin affect the MEHP levels (Cole et al., 1981). The highest levels of MEHP (up to 300 μg/ml, which are above those exerting inhibitory activity in our experiments) were found in plasma products shipped at ambient temperatures. Although protein binding might reduce the biological activity of MEHP in stored blood, our results indicate that products containing this substance may affect vascular and other smooth muscles when infused into patients. Furthermore, the blood content of DEHP correlates with the micro-aggregation of platelets in dog blood stored in plastic bags (Rubin and Jaeger, 1973), so raising the question of whether DEHP and MEHP affect human platelets.

Acknowledgements

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VISUALIZATION OF LHRH RECEPTORS IN THE RAT BRAIN

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In addition to its property to stimulate LH and FSH release from pituitary gonadotrophs, luteinizing hormone-releasing hormone (LHRH) has been shown to possess various extrapituitary actions (McCann, 1982). Both rat testis and ovary possess specific LHRH receptors (Clayton and Catt, 1981). Immunohistochemical staining shows the presence in the rat CNS of LHRH reactive neurons in various regions (Krey and Silvermann, 1984). Furthermore, intracerebral injections of LHRH or LHRH analogs in various regions produce strong effects on rat sexual behavior (McCann, 1982). Such data suggest a specific role of LHRH in the CNS, possibly as neurotransmitter or neuromodulator. Despite extensive characterization of the pituitary LHRH receptors (Clayton and Catt, 1981) the presence of LHRH receptors in the CNS could never be documented. This report describes the successful autoradiographic visualization of LHRH receptors in selected brain areas, particularly in the hippocampus.

[D-Ala^6,N$_\alpha$MeLeu^7,Pro^{9}NEt]LHRH (kindly provided by Dr. J. Rivier, San Diego) was iodinated and purified according to Perrin et al. (1983). The ligand has been shown to label specific and high affinity LHRH receptors in homogenates of rat anterior pituitary (Perrin et al., 1983) and was tested in the present studies in preliminary in vitro binding experiments for its specificity to label pituitary LHRH receptors.

Cryostat sections (10 μm) of male Sprague-Dawley rats (approximately 200-250 g) were preincubated for 10 min in Tris-HCl buffer (50 mM, pH 7.4) containing $CaCl_2$ (2 mM) and KCl (5 mM) at 21°C then washed twice for 2 min in the same buffer without additional salts added. Incubation was carried out for 2 h at 4°C in Tris-HCl buffer (170 mM, pH 7.4) containing bovine serum albumin (1%) and iodinated ligand (0.16×10^6 dpm/ml, ca. 80 pM). Non-specific binding was determined in the presence of 1 μM unlabeled [D-Ala^6,Pro^{9}NEt]LHRH. At the end of the incubation period, the slides were washed twice for 2 min in cold, ligand-free buffer. The dried sections were juxtaposed with tritium-sensitive film (Ultrofilm, LKB Instruments) and exposed for 7-14 days.

The rat hippocampal formation is highly enriched in [^{125}I][D-Ala^6,N$_\alpha$MeLeu^7,Pro^{9}NEt]LHRH receptors (fig. 1A). The CA_1, CA_2 and particularly the CA_3 region, but not the dentate gyrus, were labeled. Moderate amounts of binding sites could also be seen in the amygdala, the septal area and the perirhinal cortex (not shown). No particular accumulation was seen in the preoptic area and the spinal cord. In comparison to the clustered labeling of the pituitary gonadotrophs (fig. 1C) the hippocampal area has a lower density of binding sites. The fact that CNS and pituitary LHRH binding sites are visualized with the same radioligand suggests that central and pituitary LHRH receptors have common binding characteristics. This is of interest since it has been suggested that there could be more than one form of endogenous LHRH (Krey and Silvermann, 1984).

This report is the first description of LHRH receptors in the mammalian CNS. The presence of LHRH receptors in septum and amygdala can be well correlated with the anatomical localization as well as the physiological role of LHRH. The high

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