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The following draft report is being provided to you for your information: "Di-(2-ethylhexyl) Phthalate (DEHP) and Metabolites of DEHP Study of Effect on Embryonic Development of the Rat *In Vitro*"

This study has many design flaws and problems (such as the positive and negative controls did not work) that make interpretation of the results difficult to understand with respect to potential biological risk. However, we feel that it should be provided to you for your information. We will provide a copy of the final report to you as soon as it becomes available.

Very truly yours,

Karen R. Miller

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**DI-(2-ETHYLHEXYL) PHTHALATE (DEHP) AND METABOLITES OF DEHP
STUDY OF EFFECT ON EMBRYONIC DEVELOPMENT OF THE RAT *IN VITRO***

CONFIDENTIAL

ECF002/990059

**PHOTOCOPY
FOR AUTHOR'S FINAL
APPROVAL**

Date: 29 January 2001 **V.4.**

**DI-(2-ETHYLHEXYL) PHTHALATE (DEHP) AND METABOLITES OF DEHP
STUDY OF EFFECT ON EMBRYONIC DEVELOPMENT OF THE RAT *IN VITRO***

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COMPLIANCE WITH GOOD LABORATORY PRACTICE

**DI-(2-ETHYLHEXYL) PHTHALATE (DEHP) AND METABOLITES OF DEHP
STUDY OF EFFECT ON EMBRYONIC DEVELOPMENT OF THE RAT *IN VITRO***

The study described in this report generally followed Good Laboratory Practice Principles, however it did not comply with all of the requirements of formal GLP guidelines. I consider the data generated to be valid.

.....
H.C. Bowden, B.Sc., C.Biol., M.I.Biol.
Study Director
Huntingdon Life Sciences Ltd.

.....
Date

CONTRIBUTING SCIENTISTS

**DI-(2-ETHYLHEXYL) PHTHALATE (DEHP) AND METABOLITES OF DEHP
STUDY OF EFFECT ON EMBRYONIC DEVELOPMENT OF THE RAT *IN VITRO***

STUDY MANAGEMENT

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SUMMARY

Procedures

The influence of the main metabolites of di-(2-ethylhexyl)phthalate (DEHP) upon growth and development *in vitro*, was assessed in Day 9 embryos from rats of the CD strain. The materials tested were mono-2-ethyl-1-hexylphthalate (MEHP), metabolites I, V, VI and IX.

A dose range finding assay was conducted using MEHP in the absence of metabolic activation. MEHP was initially added to the culture medium at nominal concentrations of 0.1, 1, 10 and 100 $\mu\text{mole/ml}$. Further concentrations of 0.08, 0.4, 2 and 10 $\mu\text{mole/ml}$ were also assessed to define the response more precisely.

In part 1 of the main study MEHP was added to the culture medium at nominal concentrations of 0.01, 0.04, 0.2, 1, 5 and 10 $\mu\text{mole/ml}$, in both the presence and absence of metabolic activation. S-9 metabolic activation mixes were prepared from animals treated with arochlor 1254 or DEHP. Cyclophosphamide (0.0179 $\mu\text{mole/ml}$) was used to demonstrate the effectiveness of the metabolic activating systems. Negative control data for comparison was generated by embryos cultured in the presence of the vehicle alone. Analysis of culture medium showed the achieved concentration for the highest level to be in the range 1.78 - 9.37 $\mu\text{mole/ml}$.

In part 2 of the main study metabolites I, V, VI and IX were each added to the culture medium at nominal concentrations of 0.002, 0.01, 0.04, 0.2, 1 and 5 $\mu\text{mole/ml}$ in the absence of metabolic activation. Negative control data for comparison was generated by embryos cultured in the presence of the vehicle alone. Analysis of culture medium showed the maximum achieved concentrations did not exceed 0.863 $\mu\text{mole/ml}$.

In Part 3 of the study DEHP and the metabolites 2-ethyl hexanol and 2-ethyl hexanoic acid were investigated. Initially a dose range finding assay was conducted; each material was added to the culture medium at nominal concentrations of 1, 5, 10 and 20 $\mu\text{mole/ml}$. Each material was further investigated at nominal concentrations of 0.04, 0.2, 1.0 and 5.0 $\mu\text{mole/ml}$. Metabolic activation was not included. Due to difficulties experienced with solubility and formulation of the test materials, this latter part of the experiment was repeated using an alternative formulation procedure. C^{14} - labelled DEHP was incorporated into the dose solutions on the first day of dosing to aid in the assessment of achieved concentrations. Negative control data for comparison was generated by embryos cultured in the presence of the vehicle alone. Analysis of culture medium showed the achieved concentration for the highest levels to be in the ranges 1.26-4.57, 0.99-1.638 and 4.364-5.13 $\mu\text{mole/ml}$ for DEHP, 2-ethyl hexanol and 2-ethyl hexanoic acid respectively.

All embryos were evaluated after approximately 48 hours in culture. Data was compiled according to the achieved concentrations for discussion and interpretation of the results.

Conclusion

It was observed in this exploratory investigation that embryos cultured in the presence of MEHP at concentrations in the region of 0.6 - 1.0 $\mu\text{mole/ml}$ exhibited adverse effects on embryonic growth, development and morphology *in vitro* both in the presence and absence of metabolic activation; slight effects were apparent at lower concentrations in the presence of DEHP-derived S-9. Generally more effects were apparent in the presence of metabolic activation, the DEHP-derived S-9 producing slightly greater effects than the arochlor-derived S-9. At concentrations of 4 $\mu\text{mole/ml}$ and above embryos had failed to survive to the end of the treatment period, both in the presence and absence of metabolic activation.

Embryos cultured in the presence of Metabolite I exhibited only marginal adverse effects on embryonic development at concentrations of 0.023-0.029 μ mole/ml, adverse effects on morphology were apparent at 0.006 μ mole/ml and above.

Embryos cultured in the presence of Metabolite V exhibited no adverse effects on embryonic growth or development *in vitro* at concentrations up to 0.408 μ mole/ml. Slight adverse effects on embryonic morphology were apparent at 0.076 μ mole/ml these were increased at 0.399 μ mole/ml and above.

Embryos cultured in the presence of Metabolite VI exhibited no adverse effects on embryonic growth, development or morphology *in vitro* at concentrations up to 0.091 μ mole/ml. At 0.3 μ mole/ml only a marginal effect on embryonic morphology was observed.

Embryos cultured in the presence of Metabolite IX exhibited no adverse effects on embryonic growth or development *in vitro* at concentrations up to 0.05 μ mole/ml, slight adverse effects were observed at 0.17 μ mole/ml. Adverse effects on morphology were apparent at 0.0004 μ mole/ml and above.

The types of malformations observed in this study were also seen in a previous study using DEHP (Study Number ECF/001), in particular abnormalities of the otic system and fore limb buds.

A comparison of the data for each metabolite suggests the following order of potential toxicity:

IX > V > VI > I > MEHP

Embryos cultured in the presence of DEHP exhibited no adverse effects on growth, development or morphology at concentrations up to 0.19 μ mole/ml. Adverse effects on embryonic growth and morphology were apparent at 0.85 μ mole/ml and above, whilst adverse effects on development were apparent at 2.0 μ mole/ml and above.

Embryos cultured in the presence of 2-ethyl hexanol at concentrations up to 0.09 μ mole/ml exhibited no adverse effects on growth, development or morphology. A slight adverse effect on development was apparent at 0.16 - 0.23 μ mole/ml. Adverse effects on embryonic growth, development and morphology were apparent at 0.99 μ mole/ml, in addition half of the embryos had failed to survive to the end of the treatment period.

Embryos cultured in the presence of 2-ethyl hexanoic acid at concentrations up to 1.1 μ mole/ml exhibited no adverse effects. At 4.364 - 5.13 μ mole/ml adverse effects on growth, development and morphology were apparent.

A comparison of these data suggest the following order of potential toxicity:

DEHP = 2-ethyl hexanol > 2-ethyl hexanoic acid

Lowest concentration (μ mole/ml) at which effects were observed:

Material	Growth		Development		Morphology
	Observable	Statistically significant	Observable	Statistically significant	
MEHP + Arochlor S-9	0.61	0.61	0.61	0.61	0.61
MEHP + DEHP S-9	0.24	0.75	0.24	0.75	0.24
MEHP	1	1	1	1	1
Metabolite I	>0.86	>0.86	0.02	>0.86	0.006
Metabolite V	>0.4	>0.4	>0.4	>0.4	0.076
Metabolite VI	>0.33	>0.33	>0.33	>0.33	0.3
Metabolite IX	0.17	>0.2	>0.2	>0.2	0.0079
DEHP	0.85	0.85	0.85	2	0.85
2-ethyl hexanoic acid	0.99	0.99	0.16	0.16	0.99
2-ethyl hexanoic acid	4.	4.3	4.3	4.3	4.3

INTRODUCTION

The aim of this study was to determine the potential toxic effects of the main di-(2-ethylhexyl)phthalate (DEHP) metabolites (MEHP, metabolite I, V, VI and IX) upon the growth and development of Day 9 rat embryos *in vitro*. In addition potential effects of DEHP were compared with those of 2-ethyl hexanol and 2-ethyl hexanoic acid.

The roller bottle culture technique was used (New, 1978). Embryos were cultured in the presence of each test material for a period of approximately 48 hours at a temperature of 38.0 ± 1.0 °C. Embryotoxic potential was assessed from effects on the survival, overall growth and development of embryos, and teratogenic potential was assessed from effects on the morphology of embryos.

The study was conducted at Huntingdon Life Sciences, Eye, Suffolk, IP23 7PX. The first embryos were allocated to the study on 14 December 1998 and experimental work was completed on 12 July 2000. Original data pertaining to this study and a copy of the final report have been stored in the Archive at Huntingdon Life Sciences.

MATERIALS AND METHODS

ANIMAL HUSBANDRY

Animals

Adult virgin female rats of the CD strain (Sprague Dawley origin) were obtained from Charles River U.K. Limited, Margate, Kent. They were allowed at least one week's acclimatisation before being paired with stock males from the same strain and source. During the first week of the acclimatisation period they were inspected daily to check their physical condition.

Environmental control

The animals were housed inside a barriered, limited-access rodent facility. All personnel entering the facility were required to shower and change into clean protective clothing.

The animal room had its own supply of filtered air, which was passed to the atmosphere without recirculation, providing at least 15 room air changes per hour. The temperature and relative humidity in the animal room were recorded daily and the records retained. Target temperature was 21°C (acceptable limits 19-23°C), and humidity was 55% (acceptable limits 40-70%).

The animals were subjected to a 12 hour light : 12 hour dark cycle (light 0100-1300 hours GMT).

Water supply

Tap water from the public supply was freely available to the animals via polyethylene or polycarbonate bottles with sipper tubes; in England the supply and quality of this water are governed by the Department of the Environment regulations. Certificates of analysis are routinely received from the supplier (Essex and Suffolk Water Company). At approximately six-month intervals, water was sampled for analysis by a laboratory independent of the supplier for lead, cadmium, polychlorinated biphenyls, organochlorine and organophosphate pesticides, and coliforms. Copies of the relevant certificates have been filed in the Archives of Huntingdon Life Sciences.

Diet

The rats were allowed free access to a commercially available laboratory animal diet (SDS Laboratory Animal Diet No.1). The supplier (Special Diets Services Limited, Witham, Essex, England) provided a Certificate of Analysis with every batch. At approximately six-month intervals diet was sampled for analysis by a laboratory independent of the supplier. Copies of the relevant certificates have been filed in the Archives of Huntingdon Life Sciences.

Contaminants

No contaminants were expected to be present in water or diet at levels known to be capable of interfering with the progress and outcome of this study.

Caging

Rats were housed in TR18 cages from Arrowmigh Biosciences, Hereford, England. The cages consisted of stainless steel bodies with stainless steel mesh lids and stainless steel grid floors and were suspended in batteries over trays covered with absorbent crêpe-paper which was replaced twice weekly and daily during mating.

At various stages of the study the maximum number of rats per cage was:

Stage	Number of rats		Cage type
	M	F	
Acclimatisation	-	up to 5	TR18
Mating	1	1	TR18
Gestation	-	up to 5	TR18

Mating procedure

Females were paired on a one-to-one basis with stock males of the same strain and source. Each morning following pairing, the trays beneath the cages were checked for ejected copulation plugs. Where a copulation plug was not found a vaginal smear was prepared and examined for the presence of spermatozoa.

The day on which evidence of mating was found was designated Day 0 of gestation.

EMBRYO CULTURE**Culture medium**

The culture medium consisted of homologous rat serum, heat-inactivated on the day of use at $56.0 \pm 0.5^\circ\text{C}$ for 40 minutes.

Metabolic activation

Where incubation was conducted in the presence of an S-9 activating system, S-9 was added to the culture medium to give a concentration of $8.0 \mu\text{l/ml}$. The co-factors NADP and glucose-6-phosphate were added to give final concentrations of 0.5 mM and 5.0 mM , respectively.

S-9 was derived from the livers of non-pregnant female rats from Harlan Olac Limited, pre-treated with Arochlor 1254 or DEHP. S-9 fractions were tested for protein content and efficacy.

Prior to use, the culture medium/S-9 mix was sterilised using a $0.2 \mu\text{m}$ pore size membrane filter (Sartorius Limited).

Culture methods

Dams were anaesthetised with a mixture of isofluorane, nitrous oxide and oxygen on the afternoon of the 9th day of gestation and, after removal of the uterus, were killed by exsanguination and/or cardiac section. For the repeat of Part 3 of the study dams were anaesthetised with isofluorane and oxygen only. Decidua were subsequently released from the uterus and the embryos dissected out. The parietal yolk sac was torn open and removed. Only overtly healthy embryos with the visceral yolk sac and ecto-placental cone intact were cultured.

Embryos were incubated at 38.0 ± 1.0 °C in 30 ml glass bottles which were rotated continuously at 60 rev/min throughout the period of culture. Each bottle contained up to five embryos with at least 1 ml of culture medium per embryo and a gas phase. The gas phase was 5% O₂, 5% CO₂ and 90% N₂ for approximately 20 hours followed by 20% O₂, 5% CO₂ and 75% N₂. Each bottle was identified by indelible marker pen showing group number, compound name and dose level. Cultures were terminated after approximately 48 hours.

Test and control materials

The test materials used in this study were:

500g	di-(2-ethylhexyl)phthalate (DEHP)	received on 13 July 1998.
10g	mono-2-ethyl-1-hexylphthalate (MEHP)	batch MOP4-1 received on 23 September 1998.
120mg	metabolite I (in diethyloxide)	received on 16 October 1998.
170mg	metabolite V (in diethyloxide)	received on 16 October 1998.
142mg	metabolite VI (in diethyloxide)	received on 16 October 1998.
128mg	metabolite IX (in diethyloxide)	received on 16 October 1998.
500g	di-(2-ethylhexyl)phthalate (DEHP)	received on 23 March 2000.
2.5ml	di-(2-ethylhexyl)phthalate (DEHP) (in acetone)	received on 16 June 2000.

The batch identification, chemical identity, purity and stability of the experimental compounds supplied for the study were the responsibility of the Sponsor.

DEHP and MEHP were stored at ambient temperature. Metabolites I, V, VI and IX were stored at 4°C and protected from light.

The positive control, cyclophosphamide was obtained from Asta Medica Ltd (Endoxana^R, Lot 605326C).

Cyclophosphamide was stored at 0-4°C.

2-ethylhexanol	Lot 60708007
2-ethylhexanoic acid	Lot 31921 913007

2-ethylhexanol and 2-ethylhexanoic acid were obtained from Sigma-Aldrich Company Ltd and were stored at ambient temperature.

TREATMENT**Dose range finding assay**

Embryos were non-selectively assigned to the following treatment groups:

Group	Treatment	Nominal concentration (μ mole/ml)	Embryo number
P1	Control	0	1-5
P2	MEHP	0.1	6-10
P3	MEHP	1	11-15
P4	MEHP	10	16-20
P5	MEHP	100	21-25

Resulting data were not considered suitable to define the dose levels for use in the main study. Consequently further treatment levels were tested as agreed by the Sponsor and documented in a file note retained with the raw data. No protocol amendment was issued. Embryos were non-selectively assigned to the following treatment groups:

Group	Treatment	Nominal concentration (μ mole/ml)	Embryo number
P1B	Control	0	26-30
P2B	MEHP	0.08	31-35
P3B	MEHP	0.4	36-40
P4B	MEHP	2	41-45
P5B	MEHP	10	46-50

Solutions of MEHP in dimethyl formamide diluted to 10% v/v with sterile deionised water were prepared immediately prior to use. No correction factors were applied to account for the volume of MEHP present. All test solutions included an oily suspension, which adhered to the glass vials and pipette tips, resulting in difficulty during dilution and dosing. For groups P1 - P5, 25 μ l were added to each 1 ml of culture medium at the beginning of the culture period. This dose volume was reduced to 10 μ l per each 1 ml of culture medium for groups P1B - P5B. The vehicle, 10% dimethyl formamide was added to the negative control group of embryos.

Main study

Concentration levels for use in the main study were determined from the results of the dose range finding assays.

Part 1

Embryos were non-selectively assigned to the following treatment groups:

Group	Treatment	Nominal concentration (μ mole/ml)	Embryo number
1	Control +	0	51-60
2	Control x	0	61-70
3	Control	0	71-80
4	MEHP +	0.01	81-90
5	MEHP +	0.04	91-100
6	MEHP +	0.2	101-110
7	MEHP +	1	111-120
8	MEHP +	5	121-130
9	MEHP +	10	131-140
10	MEHP x	0.01	141-150
11	MEHP x	0.04	151-160
12	MEHP x	0.2	161-170
13	MEHP x	1	171-180
14	MEHP x	5	181-190
15	MEHP x	10	191-200
16	MEHP	0.01	201-210
17	MEHP	0.04	211-220
18	MEHP	0.2	221-230
19	MEHP	1	231-240
20	MEHP	5	241-250
21	MEHP	10	251-260
22	Cyclophosphamide +	0.0179	261-270, 281-284
23	Cyclophosphamide x	0.0179	271-280, 285-287

- + Cultured in the presence of S-9 metabolic activating system derived from arochlor-treated non-pregnant female rats.
- x Cultured in the presence of S-9 metabolic activating system derived from DEHP-treated non-pregnant female rats.

Solutions of MEHP in dimethyl formamide diluted to 10% v/v with sterile deionised water were prepared immediately prior to use. No correction factors were applied to account for the volume of MEHP present. Test solutions in the range 0.02 to 1 mmole (giving final concentrations of 0.2 to 10 μ mole/ml) included an oily suspension, which adhered to the glass vials and pipette tips, resulting in difficulty during dilution and dosing. Cyclophosphamide was dissolved in sterile deionised water. 10 μ l were added to each 1 ml of culture medium/S-9 mix at the beginning of the culture period. A similar volume of vehicle was added to the negative control group of embryos.

Part 2

Embryos were non-selectively assigned to the following treatment groups:

Group	Treatment	Nominal concentration (μ mole/ml)	Embryo number
24	Control	0	288-307, 548-552
25	I	0.002	308-317
26	I	0.01	318-327
27	I	0.04	328-337
28	I	0.2	338-347
29	I	1	348-357
30	I	5	358-367
31	V	0.002	368-377
32	V	0.01	378-387
33	V	0.04	388-397
34	V	0.2	398-407
35	V	1	408-417
36	V	5	418-427
37	VI	0.002	428-437
38	VI	0.01	438-447
39	VI	0.04	448-457
40	VI	0.2	458-467
41	VI	1	468-477
42	VI	5	478-487
43	IX	0.002	488-497
44	IX	0.01	498-507
45	IX	0.04	508-517
46	IX	0.2	518-527
47	IX	1	528-537
48	IX	5	538-547

Each metabolite was supplied in two vials as solutions in diethyloxide. The diethyloxide was evaporated using a nitrogen flow until the volume remaining in each vial was approximately 0.1 ml. Dimethyl formamide was added to the remaining solutions and further diluted to 10% v/v with sterile deionised water. No correction factors were applied to account for the purity or volume of metabolites or any residual diethyloxide present. Test solutions were generally prepared immediately prior to use. However, on two occasions insufficient embryos were obtained to complete the dosing in a single day, therefore further test solutions were prepared at a later date from the 0.5mmole solution which had been stored at approximately -20°C. Slight oily suspensions were only observed in the 0.5mmole solutions of metabolites V, VI and IX. 10 μ l were added to each 1 ml of culture medium at the beginning of the culture period. A similar volume of vehicle was added to the negative control group of embryos.

Part 3**Dose range finding assay**

Embryos were non-selectively assigned to the following treatment groups:

Group	Treatment	Nominal concentration (μ mole/ml)	Embryo number
49	Control	0	533-557
50	DEHP	1	558-562
51	DEHP	5	563-567
52	DEHP	10	568-572
53	DEHP	20	573-577
54	2-ethylhexanol	1	578-582
55	2-ethylhexanol	5	583-587
56	2-ethylhexanol	10	588-592
57	2-ethylhexanol	20	593-597
58	2-ethylhexanoic acid	1	598-602
59	2-ethylhexanoic acid	5	603-607
60	2-ethylhexanoic acid	10	608-612
61	2-ethylhexanoic acid	20	613-617

Main study

Concentration levels for use in the main study were determined from the results of the dose range finding assay. Embryos were non-selectively assigned to the following treatment groups:

Group	Treatment	Nominal concentration (μ mole/ml)	Embryo number
62	Control	0	618-627
63	DEHP	0.04	628-637
64	DEHP	0.2	638-647
65	DEHP	1.0	648-657
66	DEHP	5.0	658-667
67	2-ethylhexanol	0.04	668-677
68	2-ethylhexanol	0.2	678-687
69	2-ethylhexanol	1.0	688-697
70	2-ethylhexanol	5.0	698-707
71	2-ethylhexanoic acid	0.04	708-717
72	2-ethylhexanoic acid	0.2	718-727
73	2-ethylhexanoic acid	1.0	728-737
74	2-ethylhexanoic acid	5.0	738-747

Solutions of each test material in dimethyl formamide diluted to 10% v/v with sterile deionised water were prepared immediately prior to use. No correction factors were applied to account for the volume of test material present. All solutions of DEHP and those of 2-ethylhexanol and 2-ethylhexanoic acid at concentrations of 0.1 and 0.5 mmole/ml included an oily suspension, resulting in difficulty during dilution and dosing. 10 µl were added to each 1 ml of culture medium at the beginning of the culture period. A similar volume of vehicle was added to the negative control group of embryos.

Part 3 repeat

Due to difficulties experienced during formulation of the test materials Part 3 main study was repeated. Embryos were non-selectively assigned to the following treatment groups:

Group	Treatment	Nominal concentration (µmole/ml)	Embryo number
75	Control	0	748-757
76	DEHP	0.04	758-767
77	DEHP	0.2	768-777
78	DEHP	1.0	778-787
79	DEHP	5.0	788-797
80	2-ethylhexanol	0.04	798-807
81	2-ethylhexanol	0.2	808-817
82	2-ethylhexanol	1.0	818-827
83	2-ethylhexanol	5.0	828-837
84	2-ethylhexanoic acid	0.04	838-847
85	2-ethylhexanoic acid	0.2	848-857
86	2-ethylhexanoic acid	1.0	858-867
87	2-ethylhexanoic acid	5.0	868-877

Solutions of each test material in dimethyl formamide were prepared immediately prior to use. Concentration calculations incorporated the volume of test material present. All further dilutions were prepared using culture medium to provide the required dosing solutions. The maximum concentration of dimethyl formamide present in these dosing solutions was 0.1% v/v, therefore 5 µl dimethyl formamide was added to 5ml of culture medium for the negative control group of embryos. On the first day of dosing C¹⁴ labelled - DEHP was incorporated into the dosing solutions for groups 76 - 79. The radioactivity of these solutions was monitored prior to dosing to assess the achieved concentrations of DEHP present, data are presented in Appendix 7.

Evaluation of embryos

After termination of culture, embryos were washed in pre-warmed (38°C) saline, and the yolk sac diameter, crown-rump length and head length measured using a micrometer disk (10:100) in a focusing eyepiece. The number of somites was counted and each embryo was examined for the presence of a heartbeat and dysmorphogenesis, and the morphological score was assessed (Brown and Fabro, 1981). An example of the morphological scoring system recording sheet has been presented in Appendix 8.

Main study embryos were stored frozen in individual vials pending possible analysis of total protein content.

Medium analysis

At the end of the treatment period culture medium from dose range finding assay 2 and the main study, parts 1, 2 and 3 was stored at approximately -20°C. These samples were later sent to a laboratory nominated by the Sponsor for analysis of test material content. Analyses were performed by Ecole Nationale Supérieure de Biologie Appliquée à la Nutrition et à l'Alimentation (ENSBANA), Food Toxicology Laboratory, Campus Universitaire 1, Esplanade Erasme, F-21000 Dijon, France. Achieved concentration levels were notified to the Study Director in the form of an analytical report and are presented in the appropriate tables and appendices.

TREATMENT OF DATA

Where appropriate, data were expressed as means with standard deviations (SD) calculated according to the formula:

$$SD = \sqrt{\frac{\sum (x - \bar{x})^2}{n - 1}}$$

where x = individual values
 \bar{x} = group mean value
 n = sample size

Main study data for test materials were subjected to statistical analyses. Dependant on the heterogeneity of variance between groups, parametric tests (analysis of variance, Snedecor and Cochran 1967) followed by Williams' test (Williams' 1971/2) or non-parametric tests, (Kruskal-Wallis, Hollander and Wolfe 1973) followed by Shirley's test (Shirley 1977) were used to analyse these data, as appropriate.

All significant (i.e. $p < 0.05$) inter-group differences from the control are reported only where supported by a significant analysis of variance (i.e. $p < 0.05$).

Summaries of observed malformations have been presented. In the context of this report the term malformation or abnormal may include both dysmorphogenesis and delayed development observations. Only those malformations not commonly observed in the historical data have been considered during interpretation of the data.

Analysis of culture medium revealed variations in achieved concentrations which suggested that a more accurate interpretation of the results may be achieved by grouping the data according to actual concentrations. Hence revised tables, based on achieved concentrations, have been included for test materials MEHP, DEHP, 2-ethyl hexanol and 2-ethyl hexanoic acid. It is the results presented in these tables which have been discussed. Data for part 3 and part 3 repeat have been combined.

RESULTS

Part 1 (Tables 1-4, 5-10A and B, Appendices 1 and 2)

Dose range finding assay

All embryos cultured in the presence of MEHP at a nominal concentration of 100 μ mole/ml (Group P5) exhibited a marked reduction in crown-rump length and were grossly abnormal and dead. At a nominal concentration of 0.1 μ mole/ml (Group P2) no effects on growth or development were observed. Results observed at nominal concentrations of 1 and 10 μ mole/ml were contradictory. At 1 μ mole/ml (Group P3) embryos exhibited slight reductions in growth and development parameters whilst no effects on growth or development were observed at 10 μ mole/ml (Group P4). A number of malformations were observed at nominal concentrations upto 1 μ mole/ml. However at 10 μ mole/ml only a single abnormal allantois was observed. As these results were not considered suitable to determine dose levels for the main study a further range finding assay was performed.

At nominal concentrations of 0.08 to 2 μ mole/ml (Groups P2B to P4B) treated embryos exhibited only slight reductions in morphological score, other growth and development parameters being essentially similar to those of the control embryos (Group P1B). However increases in malformations were observed at these concentrations. At a nominal concentration of 10 μ mole/ml (Group P5B) all embryos appeared grossly abnormal and were dead.

The variation in the results seen at 10 μ mole/ml in the two dose range finding assays was thought to be probably due to the technical difficulties experienced during the formulation and dosing of MEHP.

The results of these two assays were used to aid in the dosage selection for use in the main study.

Main study

Part 1

Control

Negative control embryos (Groups 1-3) exhibited malformations at levels higher than normally expected; as illustrated by the historical control data in appendix 9. This resulted in some difficulty in interpretation of the data, however higher incidences of malformations were typically apparent in the treatment groups. The cause of the malformations seen in the control embryos is unknown but it is thought possible that there may be a background change within the strain of rat used.

Metabolic activation

Cyclophosphamide has deleterious effects upon embryonic growth, development and morphology only when cultured with metabolic activation (Fantel *et al*, 1979, *Life Sci* 25, 67-72). Embryos cultured with cyclophosphamide at 0.0179 μ mole/ml in the presence of the metabolic activating systems (Groups 22 and 23) did not exhibit the expected effects on growth (crown-rump and head lengths) and development (somite number and morphological score) parameters. Only slight increases in the incidences of morphological abnormalities were observed. However, further studies confirmed the activity of both the arochlor-derived and DEHP-derived S-9 (Appendix 11). Previous data collected at Huntingdon Life Sciences suggests that the use of 100% serum as the culture medium may afford the embryo some protection against deleterious effects.

MEHP

Analysis of culture medium showed a wide variation in the actual concentrations achieved. No analyses of culture medium were performed for nominal concentrations of 0.01 and 0.04 μ mole/ml. The achieved concentration ranges were:

		Nominal Concentration (μ mole/ml)	Achieved concentration (μ mole/ml)	
			Min	Max
MEHP	Arochlor S-9	0.2	0.04	0.07
		1	0.04	1.33
		5	0.34	1.40
		10	1.78	9.37
MEHP	DEHP S-9	0.2	0.04	0.10
		1	0.24	0.75
		5	0.12	1.56
		10	2.09	8.58
MEHP		0.2		<0.04
		1	<0.04	0.18
		5	0.22	1.03
		10	4.03	4.58

Data were revised and presented as groups with the following concentration ranges:

		Concentration range (μ mole/ml)	
		Min	Max
MEHP	Arochlor S-9	0.04	0.07
		0.34	0.34
		0.61	0.71
		1.33	1.78
		4.48	9.37
MEHP	DEHP S-9	0.04	0.07
		0.10	0.12
		0.24	0.35
		0.75	2.09
		7.87	8.58
MEHP		<0.04	<0.04
		0.18	0.22
		1.03	1.03
		4.03	4.58

Embryos cultured in the presence of MEHP, with arochlor derived S-9, at concentrations up to 0.34 μ mole/ml exhibited growth and development parameters essentially similar to those of the control embryos (Group 1). A slight but statistically significant reduction in head length and a significant reduction in morphological score were observed in the concentration range 0.61- 0.71 μ mole/ml. Significant reductions in all growth and development parameters were observed at 1.33 – 1.78 μ mole/ml. Malformations were observed at concentrations of 0.61 μ mole/ml and above. In the concentration range 4.48 – 9.37 μ mole/ml a marked reduction in yolk sac diameter was observed and all embryos appeared grossly abnormal and were dead.

Embryos cultured in the presence of MEHP, with DEHP derived S-9, at concentrations up to 0.35 μ mole/ml exhibited growth parameters essentially similar to those of the control embryos (Group

2), marginal reductions in development parameters were observed. At concentrations of 0.75 – 2.09 μ mole/ml statistically significant reductions in crown-rump and head lengths, somite number and morphological score were observed. A low incidence of abnormal posterior neuropores was observed at 0.10 – 0.12 μ mole/ml, whilst at 0.24 μ mole/ml and above further malformations were observed. In addition, at 7.87 μ mole/ml all embryos appeared grossly abnormal and were dead.

Embryos cultured in the presence of MEHP, without S-9, at concentrations up to 0.22 μ mole/ml were essentially similar to those of the control embryos (Group 3). At a concentration of 1.03 μ mole/ml a slight reduction in crown-rump length and statistically significant reductions in head length, somite number and morphological score were observed, in addition malformations were apparent. In the concentration range 4.03 – 4.58 μ mole/ml a marked reduction in yolk sac diameter was observed and all embryos appeared grossly abnormal and were dead.

The types of malformations generally observed in embryos treated with MEHP included abnormalities of the yolk sac circulation, heart, posterior neuropore, otic system and forelimb buds. Generally more effects were apparent in the presence of metabolic activation, when unfused or abnormal caudal neural tubes, hind, mid and fore brains were also observed. Inclusion of DEHP derived S-9 in the culture medium produced slightly greater effects than those of arochlor derived S-9, though further studies have shown the arochlor derived S-9 to be more active than the DEHP derived S-9.

Part 2 (Tables 11 to 18, Appendix 3)

Analysis of culture medium showed variation in the actual concentrations achieved. The achieved concentration ranges were:

	Nominal Concentration (μ mole/ml)	Achieved concentration (μ mole/ml)	
		Min	Max
Metabolite I	0.002		<0.006
	0.01		<0.006
	0.04	0.006	0.006
	0.2	0.023	0.029
	1	0.156	0.163
	5	0.744	0.863
Metabolite V	0.002		<0.002
	0.01		<0.002
	0.04	0.002	0.002
	0.2	0.014	0.014
	1	0.076	0.081
	5	0.399	0.408
Metabolite VI	0.002		<0.002
	0.01		<0.002
	0.04	0.002	0.002
	0.2	0.017	0.018
	1	0.091	0.095
	5	0.300	0.331
Metabolite IX	0.002		<0.0004
	0.01		<0.0004
	0.04	0.0004	0.0009
	0.2	0.0079	0.0097
	1	0.049	0.050
	5	0.170	0.205

It was not considered necessary to amend the grouping of the data.

Metabolite I

Embryos cultured in the presence of Metabolite I at concentrations upto $0.006\mu\text{mole/ml}$ (Groups 25 - 27) exhibited growth and development parameters essentially similar to those of the control embryos (Group 24). Marginal reductions in morphological score only were observed at $0.023\text{-}0.029\mu\text{mole/ml}$ and above (Groups 28 - 30) which were not shown to be statistically significant. Low levels of malformations were apparent at concentrations less than $0.006\mu\text{mole/ml}$ (Groups 25 and 26), but levels were not greater than seen in the negative control embryos (Group 24). Slight increases in the incidence of malformations were observed at concentrations of $0.006\mu\text{mole/ml}$ and above (Groups 27 - 30), which included abnormalities of the heart, otic system and forelimb buds and unfused or abnormal, hind, mid and fore brains.

Metabolite V

Embryos cultured in the presence of Metabolite V at concentrations upto $0.108\mu\text{mole/ml}$ (Groups 31 - 36) exhibited growth and development parameters essentially similar to those of the control embryos (Group 24). Slight increases in the incidences of malformations were observed at concentrations upto $0.014\mu\text{mole/ml}$ (Groups 31 - 34), which were not concentration-related. These were further increased at a concentration of $0.076\mu\text{mole/ml}$ and above (Groups 35 and 36). Observed malformations included those of the heart, otic system, forelimb buds and caudal neural tube and unfused or abnormal hind brains.

Metabolite VI

Embryos cultured in the presence of Metabolite VI at concentrations upto $0.331\mu\text{mole/ml}$ (Groups 37 - 42) exhibited growth and development parameters essentially similar to those of the control embryos (Group 24). Low incidences of malformations were observed in all treatment groups but were generally not concentration - related. However malformations of the otic system and forelimb buds were observed at a higher incidence at $0.3\text{-}0.331\mu\text{mole/ml}$ (Group 42) and may have been related to treatment.

Metabolite IX

Embryos cultured in the presence of Metabolite IX at concentrations upto $0.05\mu\text{mole/ml}$ (Groups 43 - 47) exhibited growth and development parameters essentially similar to those of the control embryos (Group 24). Slight reductions in growth parameters were apparent at concentrations of $0.17\text{-}0.205\mu\text{mole/ml}$ (Group 48), which were not shown to be statistically significant. Malformations were observed at concentrations upto $0.0009\mu\text{mole/ml}$ (Groups 43 and 44) which were at similar levels to those seen in the negative control embryos (Group 24). Slight increases in the incidences of several malformations were observed at concentrations of $0.0079\mu\text{mole/ml}$ and above (Groups 45 - 48), which included abnormalities of the heart, caudal neural tube, mid brain, otic system and forelimb buds and unfused or abnormal hind brains.

Part 3 (Tables 19 to 30, 31-36A and B; Appendices 4 to 6)**Dose range finding assay**

Embryos cultured in the presence of DEHP at nominal concentrations up to 20 $\mu\text{mole/ml}$ (Groups 50 to 53) exhibited growth and development parameters essentially similar to those of the Control embryos (Group 49). Malformations were observed in all groups which were generally not concentration-related; increases above Control levels were apparent for abnormal yolk sac circulation at a nominal concentration of 5 $\mu\text{mole/ml}$ and above and abnormal otic system at 10 $\mu\text{mole/ml}$ and above.

Embryos cultured in the presence of 2-ethyl hexanol at nominal concentrations up to 5 $\mu\text{mole/ml}$ (Groups 54 and 55) were essentially similar to Control embryos (Group 49). Isolated incidences of abnormal heart and blood in the brain cavities and neuropore were observed at 5 $\mu\text{mole/ml}$; however these were not seen at higher concentrations. Marked effects on embryonic growth, development and morphology were apparent at a nominal concentration of 10 $\mu\text{mole/ml}$ (Group 56), in addition two embryos were dead. At a nominal concentration of 20 $\mu\text{mole/ml}$ (Group 57) all embryos had failed to survive and showed no development beyond that at the start of the culture period.

Embryos cultured in the presence of 2-ethyl hexanoic acid at a nominal concentration of 1 $\mu\text{mole/ml}$ (Group 58) exhibited growth and development parameters essentially similar to those of the Control embryos (Group 49). Slight effects on embryonic growth and development were observed at 5 and 10 $\mu\text{mole/ml}$ (Groups 59 and 60); these effects were substantially increased at a nominal concentration of 20 $\mu\text{mole/ml}$ (Group 61). Yolk sac circulation was affected at a nominal concentration of 1 $\mu\text{mole/ml}$ (Group 58). Increases in malformations were apparent at nominal concentrations of 5 $\mu\text{mole/ml}$ and above (Groups 59 to 61), with 4 of the 5 embryos at 20 $\mu\text{mole/ml}$ classified as grossly abnormal.

These results were used to aid in the dosage selection for the main study.

Main study

Analysis of culture medium showed a wide variation in the actual concentrations achieved. The achieved concentration ranges were:

	Nominal Concentration ($\mu\text{mole/ml}$)	Achieved concentration ($\mu\text{mole/ml}$)	
		Min	Max
DEHP	0.04	0.0007	0.0009
	0.2	0.002	0.003
	1	0.019	0.019
	5	1.260	2.045
2-ethyl hexanol	0.04		<0.014
	0.2	0.014	0.016
	1	0.07	0.092
	5	1.522	1.638
2-ethyl hexanoic acid	0.04	0.010	0.012
	0.2	0.056	0.062
	1	0.389	0.410
	5	4.364	4.487

Main study repeat

The achieved concentration ranges were:

	Nominal Concentration (μ mole/ml)	Achieved concentration (μ mole/ml)	
		Min	Max
DEHP	0.04	0.03	0.04
	0.2	0.15	0.19
	1	0.85	1.13
	5	2.34	4.57
2-ethyl hexanol	0.04		<0.03
	0.2	0.03	0.06
	1	0.16	0.23
	5	0.99	1.43
2-ethyl hexanoic acid	0.04		<0.2
	0.2	0.20	0.22
	1	0.98	1.10
	5	4.80	5.13

Combined data

Data for Part 3 and Part 3 repeat were combined and presented as groups with the following concentration ranges:

	Concentration range (μ mole/ml)	
	Min	Max
DEHP	0.0007	0.003
	0.019	0.04
	0.15	0.19
	0.85	1.26
	2.045	2.34
2-ethyl hexanol	4.30	4.57
	0.014	0.016
	0.03	0.092
	0.16	0.23
	0.99	1.638
2-ethyl hexanoic acid	0.01	0.062
	0.20	0.41
	0.98	1.10
	4.364	5.13

Embryos cultured in the presence of DEHP at concentrations up to 0.19 μ mole/ml were essentially similar to those of the control group. Statistically significant reductions in crown-rump and head lengths were observed at concentration ranges of 0.85 – 2.34 μ mole/ml, slight reductions in development parameters were also observed of which only the somite number at concentrations of 2.045 – 2.34 μ mole/ml was shown to be statistically significant. At concentrations of 4.30 – 4.57 μ mole/ml statistically significant reductions in all growth and development parameters were apparent. Incidences of malformations were increased at concentrations of 0.85 μ mole/ml and above and principally included abnormalities of the yolk sac circulation, heart, caudal neural tube, posterior neuropore, hind and mid brains, otic system and forelimb buds.

Embryos cultured in the presence of 2-ethyl hexanol at concentrations up to 0.092 μ mole/ml exhibited growth and development parameters essentially similar to those of the control embryos. A

concentrations of 0.16 – 0.23 μ mole/ml a slight but statistically significant reduction in morphological score was observed. Marked, significant reductions in growth and development parameters were apparent at concentrations of 0.99 – 1.638 μ mole/ml. Low incidences of malformations were observed in concentration ranges 0.014 – 0.23 μ mole/ml which were not generally concentration-related. In the concentration range 0.99 – 1.638 μ mole/ml high incidences of malformations were observed. These included abnormal yolk sac circulation, posterior neuropores, otic system, branchial bars, forelimb buds, unfused or abnormal hind and mid brains and absent fore brains, in addition half of the embryos appeared grossly abnormal and were dead.

Embryos cultured in the presence of 2-ethyl hexanoic acid at concentrations up to 1.10 μ mole/ml exhibited growth and development parameters essentially similar to those of the control embryos. Statistically significant reductions in growth and development parameters were apparent at concentrations of 4.364 – 5.13 μ mole/ml. Low incidences of malformations were seen at concentrations up to 1.10 μ mole/ml which were not generally concentration-related. At 4.364 – 5.13 μ mole/ml high incidences of malformations were apparent, these principally included abnormal yolk sac circulation, caudal neural tube, posterior neuropore, forelimb buds, unfused hind and mid brains and absent fore brains.

CONCLUSION

It was observed in this exploratory investigation that embryos cultured in the presence of MEHP at concentrations in the region of 0.6 – 1.0 μ mole/ml exhibited adverse effects on embryonic growth, development and morphology *in vitro* both in the presence and absence of metabolic activation; slight effects were apparent at lower concentrations in the presence of DEHP-derived S-9. Generally more effects were apparent in the presence of metabolic activation, the DEHP-derived S-9 producing slightly greater effects than the arochlor-derived S-9. At concentrations of 4 μ mole/ml and above embryos had failed to survive to the end of the treatment period, both in the presence and absence of metabolic activation.

Embryos cultured in the presence of Metabolite I exhibited only marginal adverse effects on embryonic development at concentrations of 0.023-0.029 μ mole/ml, adverse effects on morphology were apparent at 0.006 μ mole/ml and above.

Embryos cultured in the presence of Metabolite V exhibited no adverse effects on embryonic growth or development *in vitro* at concentrations up to 0.408 μ mole/ml. Slight adverse effects on embryonic morphology were apparent at 0.076 μ mole/ml these were increased at 0.399 μ mole/ml and above.

Embryos cultured in the presence of Metabolite VI exhibited no adverse effects on embryonic growth, development or morphology *in vitro* at concentrations up to 0.091 μ mole/ml. At 0.3 μ mole/ml only a marginal effect on embryonic morphology was observed.

Embryos cultured in the presence of Metabolite IX exhibited no adverse effects on embryonic growth or development *in vitro* at concentrations up to 0.05 μ mole/ml, slight adverse effects were observed at 0.17 μ mole/ml. Adverse effects on morphology were apparent at 0.0004 μ mole/ml and above.

The types of malformations observed in this study were also seen in a previous study using DEHP (Study Number ECF/001), in particular abnormalities of the otic system and fore limb buds.

A comparison of the data for each metabolite suggests the following order of potential toxicity:

IX > V > VI > I > MEHP

Embryos cultured in the presence of DEHP exhibited no adverse effects on growth, development or morphology at concentrations up to 0.19 μ mole/ml. Adverse effects on embryonic growth and morphology were apparent at 0.85 μ mole/ml and above, whilst adverse effects on development were apparent at 2.0 μ mole/ml and above.

Embryos cultured in the presence of 2-ethyl hexanol at concentrations up to 0.09 μ mole/ml exhibited no adverse effects on growth, development or morphology. A slight adverse effect on development was apparent at 0.16 – 0.23 μ mole/ml. Adverse effects on embryonic growth, development and morphology were apparent at 0.99 μ mole/ml, in addition half of the embryos had failed to survive to the end of the treatment period.

Embryos cultured in the presence of 2-ethyl hexanoic acid at concentrations up to 1.1 μ mole/ml exhibited no adverse effects. At 4.364 – 5.13 μ mole/ml adverse effects on growth, development and morphology were apparent.

A comparison of these data suggest the following order of potential toxicity:

DEHP = 2-ethyl hexanol > 2-ethyl hexanoic acid

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