THE UNIVERSITY OF MICHIGAN

MEDICAL SCHOOL
Department of Biological Chemistry

Annual Report

ACCUMULATION, TOXICITY, AND METABOLISM OF COMMON PLASTICIZERS IN HUMANS

Edward A. Napier, Jr.

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PRINCIPAL INVESTIGATORS

The principal investigators responsible for the program are cited below, together with the areas of the research for which they have principal responsibility:

1. Dr. Ronald Easterling, Associate Professor of Internal Medicine, and who is in charge of the University Hospital Hemodialysis Unit, is responsible for patient care and the hemodialysis studies, both *in vitro* and *in vivo*.

2. Dr. John Groves, Assistant Professor of Chemistry, is responsible for the supervision of much of the analyses and the important synthesis of the stable isotope double-labeled phthalate esters, and other plasticizers.

3. Dr. Raymond Kahn, Professor of Anatomy, is supervising the *in vitro* metabolism and affect of these plasticizers with respect to toxicity in human cell cultures.

4. Dr. Edward A. Napier, Jr., Associate Professor of Biological Chemistry, is conducting the analytical monitoring in these studies, and is responsible for the synthesis of the double-labeled radio isotope plasticizers.

5. Dr. Harold Oberman, Professor of Pathology, and Director of the University Hospital Blood Bank, coordinates and undertakes the responsibility for the important monitoring aspects of this proposal.

6. Dr. John Penner, Professor of Internal Medicine, and Director of the Hematological Research Laboratories at the University Hospital, is responsible for patient care in addition to the supervision of the blood studies cited in the technical proposal.

SUMMARY

Phthalate esters, commonly used as plasticizers, are ubiquitous in their occurrence; but their effect on human health has yet to be ascertained.

For studies of the metabolism of phthalates in human cell cultures, double-labeled radioactive phthalates (^{14}C; ^{3}H) have been synthesized, and *in vitro* studies are now in progress. Correspondingly, double-labeled *stable* isotope esters have also been synthesized (^{13}C; ^{2}H), which will be utilized to study their metabolism *in vivo* by humans.

Our laboratories have shown, *in vitro*, that phthalates are readily leached out into solutions perfused through various plastic hemodialysis units, even if only saline is used as the perfusing solution. With lipid containing solutions, such as human plasma or corn oil, correspondingly considerably more phthalate esters are in these solutions subsequent to perfusion. Remarkably, however, in the assay of patients plasma subsequent to hemodialysis using the same units (normally for a period of 6 hr), phthalates were very difficult to detect *in vivo*, suggesting that tissues are rapidly clearing the plasma of plasticizers.

Toxicity studies with human cell cultures (WI-38 fetal diploid, fibroblasts, 50 generation ± 10) have shown a clear fatal dose-response relationship, at phthalate concentrations of 0.01 to 1.0 mM over a period of several days exposure of the cells.

In our preliminary monitoring studies of human tissues, phthalates have been detected in hearts, liver, lung, spleen and kidney; our data, however, are too preliminary at the present date to correlate the occurrence of the phthalates with patient histories.

INTRODUCTION SUMMARY

BACKGROUND

At least 8 common plasticizers have been shown to be toxic, and more specifically, to cite only 1 group, teratogenic in rats by Autian's Laboratory (J. Pharm. Sci. 61:51, 1972). These same plasticizers are found in various common plastic intravenous tubings and bags and have been reported to be released into the solutions contained within these and other plastic containers. For example, phthalate esters have been found in whole blood (Lancet II:151, 1970) or plasma (Lancet I:35, 1970) stored in plastic bags; and from perfusion studies with rat livers it appears that at least 1 phthalate ester (di-2-ethyl hexylphthalate of DEHP) is not metabolized but is rather accumulated by the liver and by human tissues after blood transfusions (Science 170: 460, 1970). Moreover, the latter plasticizer (DEHP) has also been shown to be present in bovine, rat, rabbit and dog heart mitochondria (Biochem., 10:4228, 1971).

Studies from the laboratories of Dr. Napier's group, for example, on the properties, absorption and metabolism of 2-alkylalkanoates (cf., Biochem., 5:1279, 1966; J. Lipid Res., 8:342, 1967; and Fed. Proc., 27:635, 1968), would suggest that the steric hindrance of the 2-ethyl grouping in DEHP would alter absorption rates, and most certainly not be easily hydrolized. Hence, it would be compatible with our studies and reasonable to expect that this, and similar compounds, would not be easily metabolized, but would be deposited in tissues.

Although our proposal is primarily oriented toward the heavy use of the

plasticizers in medical applications (up to 40% by weight in some plastics), it is worthy to note in passing that these same plasticizers can be found in food stuffs packed in plastics (e.g., milk). These same volatile plasticizers are also the primary cause of the "film" deposited on the inside of automoble windshields, and has led at least one manufacturer to ban the use of dioctyl phthalate in plastics used in automobile parts (this, however, represents a ban of only one plasticizer). Little is known, however, of the long-term chronic exposure of these volatile plasticizers in humans, and it is hopeful that the present proposal will contribute new information in this direction.

SUMMARY OF PROPOSAL

To study the accumulation, toxicity, and metabolism of common plasticizers in humans, both in vitro and in vivo techniques are being employed; and to note the present chronic accumulation of these plasticizers in humans, a program was initiated to monitor these plasticizers in variour normal and abnormal human tissues.

In brief, for the in vitro studies, the toxicity and metabolic fate of these plasticizers is being studied in human cell cultrues and blood cells. In the metabolic aspects of these studies carbon-14 and tritiated double-labeled esters was synthesized in our laboratories, and their metabolic fate by these tissues and cells is being followed. Another important in vitro technique in this proposal is the study of the release of the plasticizers into fluids used in hemodialysis units and in units of blood and blood products commonly employed in hospitals at the present.

In vivo studies are also planned on their absorption in humans (through

standard balance techniques); their mode of transport in the vascular system, particularly with respect to lipoprotein carriers; and their metabolic fate subsequent to both peroral and intravenous administration. These latter important studies will be undertaken with plasticizer esters synthesized with double-labeled stable, common non-radioactive isotopes to maximize patient safety (carbon-13 and deuterium-labeled esters). Through the use of mass spectrometry, it will be possible to identify and quantitate these latter isotopes and their products at an extremely low level (e.g., picogram amounts).

Another major important task in this proposal is to monitor the chronic accumulation of these plasticizers in various tissues from both normal and abnormal subjects. This will be attempted through assay on various excised tissues such as heart (at death or through transplant), kidney (transplant and excised tissues), spleen and lung. It is also expected that with patient consent, biopsies of the liver and adipose tissue may be taken, not only during laparotomy, but most particularly from hemodialysis patients and plasmapheresis donors.

Assay of Phthalate Esters:

The ubiquitous occurrence of the phthalates requires careful distillation and purification of all reagents, particularly when using sensitive techniques such as the mass spectrometer to quantitate their level.

To briefly outline our assay procedure, tissues are extracted with chloroform: methanol (2:1) essentially according to the method of Folch (cf, Saladin and Napier, J. Lipid Res. 8:342, 1967). The extracted lipids are then taken up with hexane and injected into the gas chromatograph containing a 1% SE-30 column, maintained at a temperature of 200°C.

The Retention Times under the above conditions for various phthalates, most of which were synthesized by our group, are as follows:

PHTHALATE	RETENTION TIME
Di-methyl	0.26 minutes
Di-ethyl	0.59
Di-isopropyl	0.79
Di-N-propyl	1.19
Di-iso-butyl	1.84
Di-N-butyl	2.47
Di-2-ethylhexyl	19.16
Di-octyl	31.60

Mass Spectroscopic Identification of Phthalates:

Figure 1 is the mass spectrum of di-methyl phthalate that was selected to illustrate fragmentations typical of the aromatic moiety of all the phthalates. The structures of these fragments are shown in Figure 2, whereby the principal fragment in the case of di-methyl phthalate (parent ion, M = 194) is the loss of M - 31 ($-OCH_3$) with the resultant m/e of 163; further characteristic m/e fragments are seen at 135, 121, 104, 93, 76, 65, 50, and 39.

Figure 3 illustrates the mass spectra of di-2-ethylhexyl phthalate that was detected in human plasma subsequent to perfusion through a hemodialysis unit for a period of four hours; the structures of the fragments characteristic for DEHP are shown in Figure 4. The principal fragments observed are at m/e's of 279, 167, 149, and 113 (in addition to the typical alkane fragments, and the aromatic fragmentations depicted in Figure 2). In addition to DEHP, significantly, 2-ethyl hexanol was also detected in the plasma samples (the artificial kidney employed was a Cordis Dow Model 4).

Figure 1

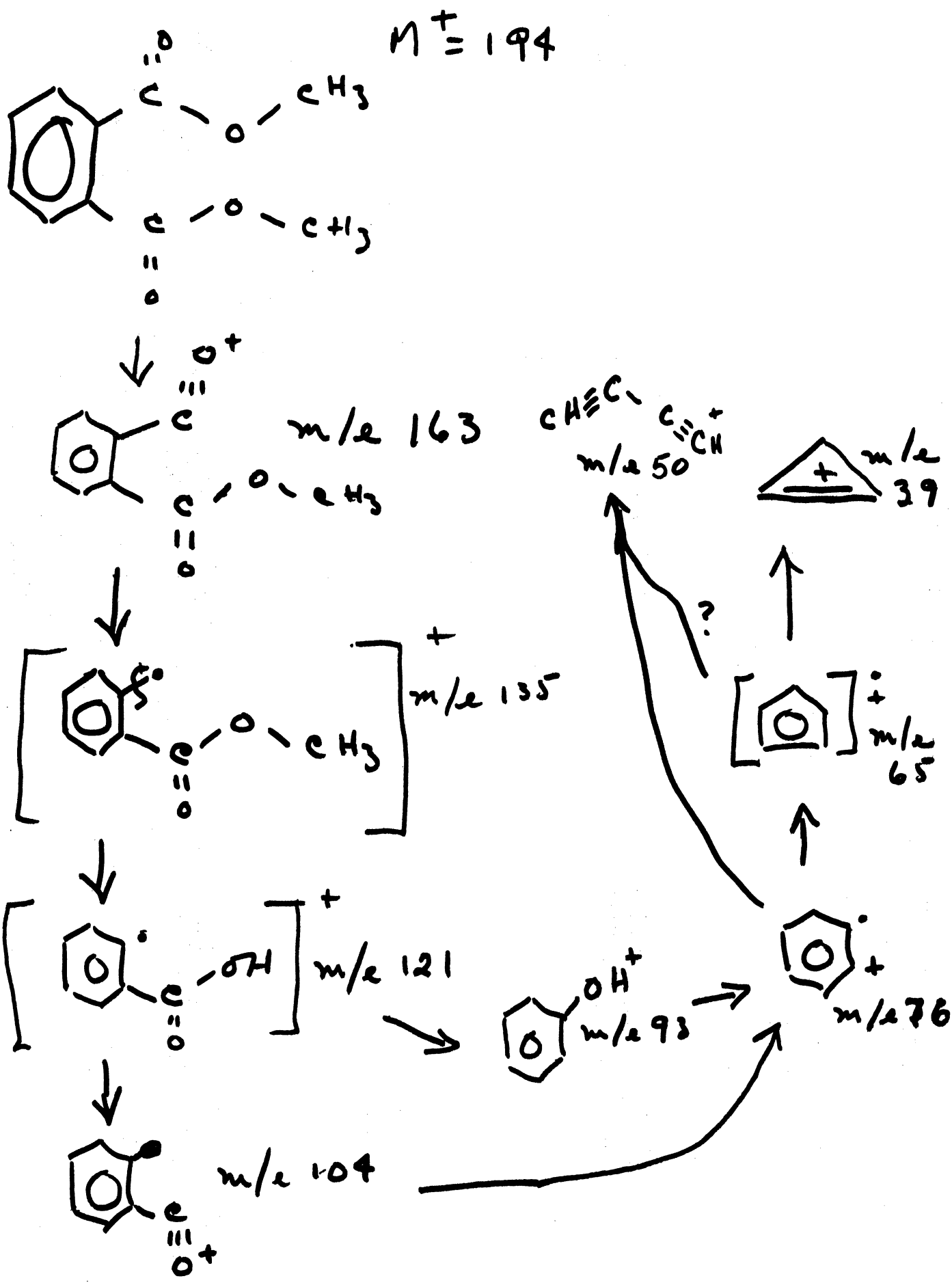

Figure 2

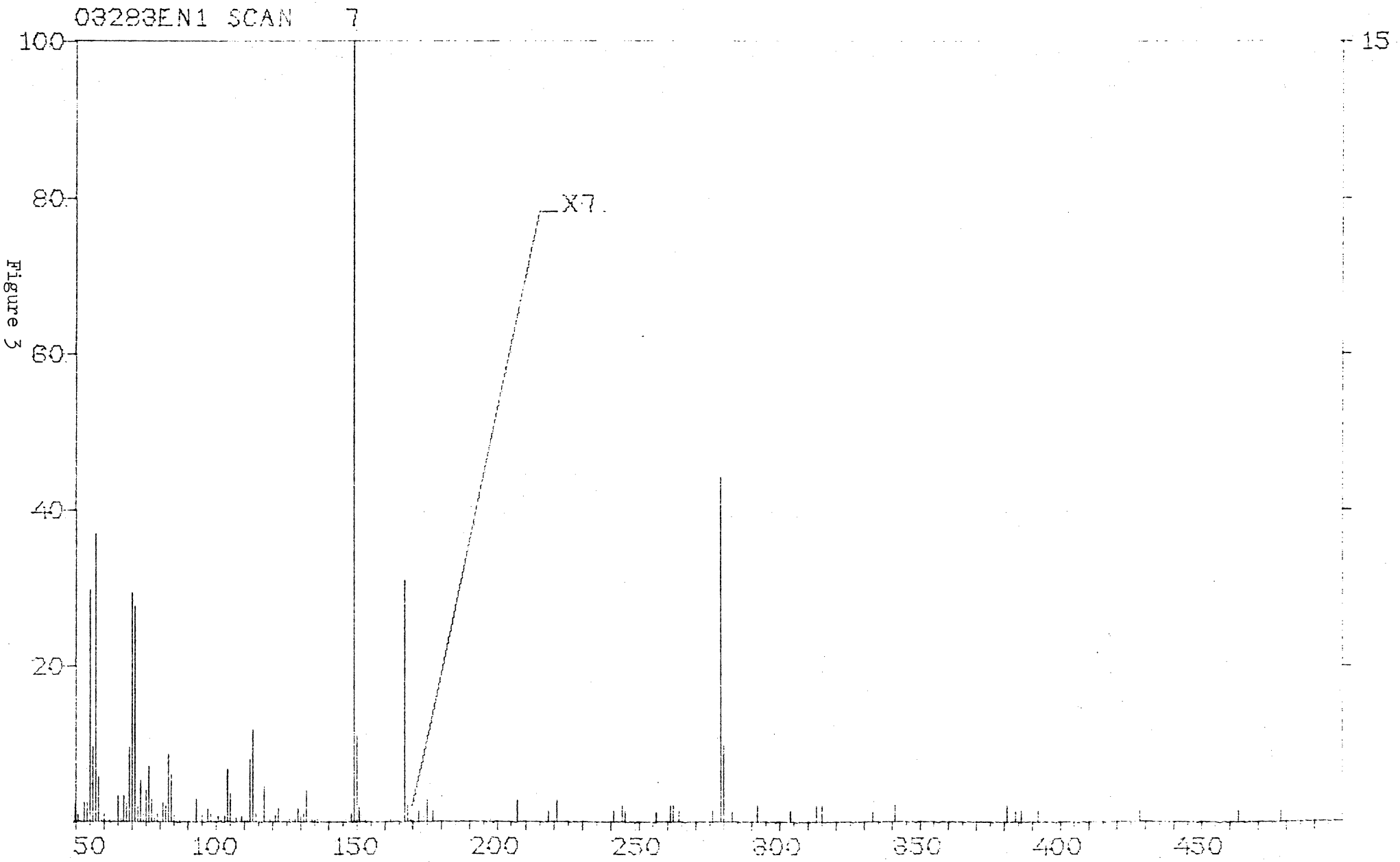
03283EN1 SCAN 7
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Figure 3

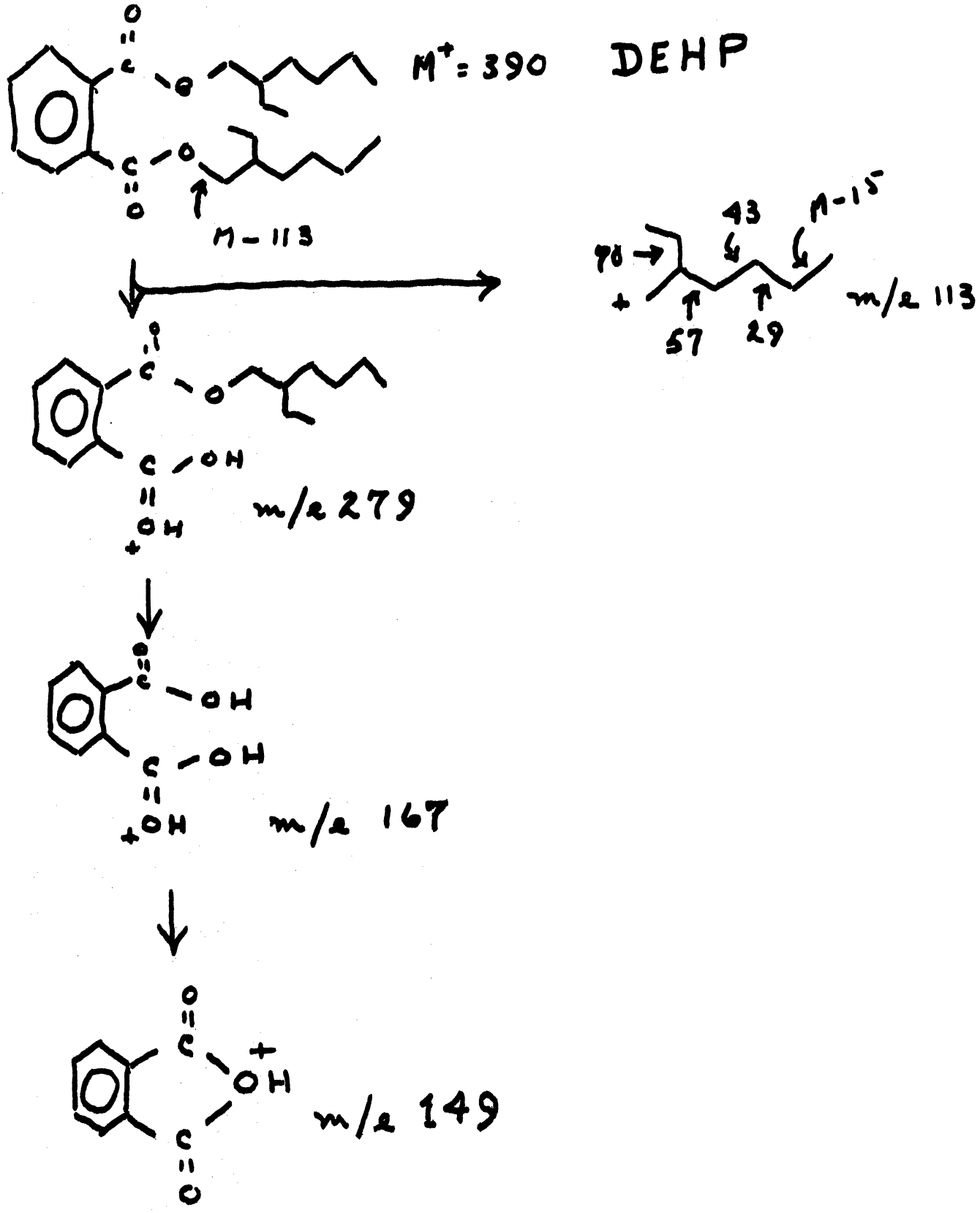


Figure 4

Characterization of Phthalate Esters:

In preparation for automated computerized identification of phthalate esters in sub-microgram quantities a series of phthalates were characterized spectroscopically, criteria of purity were established and optimal gas chromatographic parameters were determined. In several cases (e.g. di-octyl phthalate) actual chemical structure was found to differ from supplier to supplier. In such cases authentic materials were synthesized from phthalic anhydride and the corresponding alcohol. Synthetic techniques for incorporation of ^{13}C and ^{2}H double labels were perfected.

Outlined below are pertinent spectral and physical properties of a number of phthalate esters used in this study to date. These authentic materials were used to compile a standard catalog of GC-Mass Spectrometer data and were used in the cell culture studies reported below.

IDENTIFICATION OF THE STRUCTURES OF COMMERCIAL SAMPLES OF "DI-OCTYL PHTHALATE"

Source	Structure
Matheson Colman and Bell	di-n-octyl phthalate
Eastman	di-n-octyl phthalate
Aldrich	bis(2-ethylhexyl) phthalate
Sargent-Welch	bis(2-ethylhexyl) phthalate

(The above was determined by gas liquid chromatography, mass spectrometry, and comparison of NMR spectra of authentic samples synthesized by our laboratories.)

1. Dimethyl Phthalate

Source: Aldrich

Characterization by G.C.

a. 3% Q F-1 on Varaport - 5' x 1/8"
inj. 175°
col. - 100-200/6° min.
1 peak - ret. time 7.5 min.

b. 3% SE-30 on Varaport - 5' x 1/8"
100-220/10° min.
inj. 110°
1 peak ret. time 4.0 min.

G.C. - mass spec.
3% SE-30
150-220/10° min.
Separator 190°
1 peak ret. time 8.8 min.

M/e		Normalized Intensity
194	(P)	3.2
164		9
163	(base)	100
135		5
133		3
120		3
105		4
104		6
92		10
78		3
77		26
76		10

2. Di-n-Butylphthalate

Source: distilled by 212-220/25 min.

Characterization by G.C.

a. 3% Q F-1 on Varaport 5' x 1/8"
inj. 175°
col.- 100-200/6° min.
1 peak - ret. time 14.0 min

b. NMR (CCl_4)
7.7δ multiplet 2H
4.4δ triplet (J=10.0 hz) 2H
1.9-1.0 complex 7H

G.C.- mass spec.
3% SE-30
150-220/10°/min.
Separator 190°
1 peak retention 21.2 min.

M/e		Normalized Intensity
278	.4	.24
223	5	3
205	5	3
150	5	9
149	base 170	100
57	10	6
56	10	6

3. Di-i-Butylphthalate

Source: Aldrich

Characterization by G.C.

a. 3% Q F-1 on Varaport 5' x 1/8"
inj. 175
col. - 100-200/6° min.
1 peak ret. time 12.6 min.

b. 3% SE-30 on Varaport 5' x 1/8"
inj. 110°
100-220/10° min.
1 peak ret. time 8.0 min.

c. NMR (CCl_4)
7.7δ com. mult. 2H
4.1δ doub. J=8Hz 2H
2.0δ mult. 1.5 H
1.0δ doub. J=8Hz 6H

G.C. - mass spec.
3% SE-30
150—>220/10° min.
Separator 190°C
1 peak ret. time 18.8 min.

M/e		Normalized Intensity
223		4
167		3
150		9
149	base	100
57		27
56		7

4. Di-2-Ethylhexylphthalate

Source: Aldrich

Characterization by G.C.

a. 3% Q F-1 on Varaport 5' x 1/8"
inj. 175°
col. - 100-200/6° min.
1 peak ret. time 22.2 min.

b. 3% SE-30 —> Varaport 5' x 1/8"
inj. 110
col. - 100 —>250/10° min.
1 peak ret. time 13.9 min.

c. NMR (CCl_4)
7.66 comp. mult. 1H
4.26 doub. J=7Hz 1H
1.6-,8 complex 9H

G.C. - mass spec.
3% SE-30 250° isotherm.
Separator 220°
1 peak ret. time 18.5 min.

M/e	Normalized Intensity	M/e	Normalized Intensity
279		58	2
168		57	10
167		56	8
150		55	6
149	base 100		
113			
112			
84			
83			
82			
72			
71			
70			
69			

5. Butyl-carbobutoxymethylphthalate

Source: Matheson-Coleman-Bell

Characterization by G.C.

a. 3% SE-30 on Varaport 5' x 1/8"
inj. 230°
210° isothermal
2 peaks - major peak 90%
ret. time 3.0 min. (major)
1.2 min. (minor)

G.C.- mas spec.

3% SE-30
250° isothermal
220° separator
major peak - ret. time 9.8 min.

M/e	Normalized Intensity
264	5.
263	0.5.
207	8
205	3
150	9
149	100
133	13
132	4
105	4
104	4
77	14
57	12
56	9

6. Dioctylphthalate

Source: Matheson-Coleman-Bell
(practical)

Characterization by G.C.

a. 3% SE-30 on Varaport 5' x 1/8"
inj. 230
col. - 210° isothermal
1 broad peak - ret. time 11.3 min.

b. NMR (CCl_4)

7.66	comp. mult.	1H
4.36	trip. (J=6Hz)	1H
1.9-.7	complex	9H

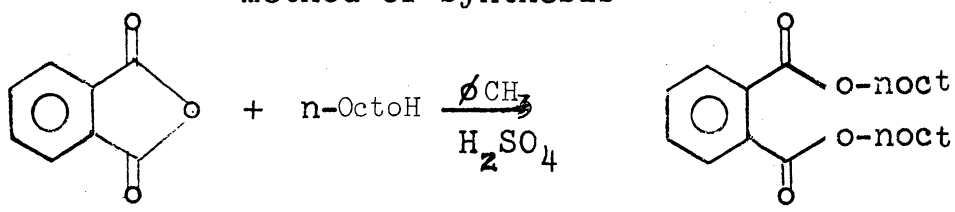
G.C. - mass spec.
3% SE-30
250° isothermal
Separator 220°
1 broad peak-ret. time 30.0 min.
(mass spec. for center of peak)

M/e		Normalized Intentisty	M/e	Normalized Intensity
280		3.7	71	4.2
167		2	70	2.9
150		9	69	2.7
149	591	100	58	.7
123		1	51	4.2
122		.8	56	3.2
117		1	55	3.0
112		1.5	54	.7
84		1.8	44	1.0
83		2.0	43	4.2
82		.8	42	2.2
			40	1.2
			41	

7. Di-n-octylphthalate

Source: Synthetic

method of synthesis



reflux overnight w/ Dean Stark trap.
Toluene and n-OCtOH removed under reduced pressure,
Remaining oil taken up in ether, washed with 5% NaOH
SotNaCl, dried over Na_2SO_4, filtered, ether removed
by H_2O pump. Product distilled; by 192-200°C at .2
mm.

Characterization by G.C.

3% SE-30 on Varaport 5' x 1/8"
inj. 230°
col. - 210° isothermal
1 broad peak - ret. time 10.9 min.
NMR (CCl_4)

7.6δ	comp. mult.	1H
4.2δ	trip. ($J=6H_z$	1H
1.9-.8	complex	9H

G.C. - mass spec.

3% SE-30
250° isothermal
Separator 220°
1 broad peak - ret. time 29.8 min.
mass spec. for center of peak.

(Continued)

8. Di-Ethylphthalate

Source: Aldrich

Characterization by G.C.

col. - 3% Q F-1 on Varaport 5' x 1/8"
inj. 175°
100-200°/6° min.
1 peak ret. time 9.5 min.
col. - 3% SE-30 on Varaport 5' x 1/8"
inj. 110°
100 - 230/10° min.
1 peak ret. time 5.3 min.

G.C. - mass spec.

3% SE-30
150° - 220/10° min.
separator 190°C
1 peak ret. time 12 min.
50 min.

M/e		Normalized Intensity
222	(P)	1.5
178		5
177		21.
176		5
150		9
149	(base)	100
122		5
121		6
106		3
105		13
104		11

(Continued)

M/e	Normalized Intensity
280	.7
167	1.9
150	9.0
149	100
123	1.4
122	1.0
113	1.2
112	1.4
84	1.9
83	2.1
82	1.0
71	6.7
70	3.3
69	2.9
58	.7
57	6.9
56	3.1
55	2.9
54	.7
44	1.0
43	5.7
42	1.7
41	2.1
40	1.2

Binding of DEHP to Human Plasma Proteins:

An important aspect of our program in the study of the metabolism and accumulation of the phthalates is to identify and quantitate which of the human plasma proteins are acting as vehicles of transport for the bound phthalates. Cohn Fraction V (albumin), low-density and high-density lipoproteins, and the residual globulins have been isolated from human plasma, and the association constants for the binding of DEHP to these plasma protein fractions are now being studied.

In preliminary studies with albumin fractionated from human plasma by the above technique, we have determined that approximately eight moles of phthalate is bound to one mole of albumin. These studies are being continued with other plasma proteins.

TISSUE CULTURE STUDIES

The emphasis of our tissue culture studies has been to study the effects of DEHP and butyl carbobutoxymethyl phthalate (often referred to as butyl glycolyl butyl phthalate, or BGBP) on cell cultures. These particular compounds were selected due to their prevalence in medical devices containing PVC. The WI-38 cells used for this study were grown in Basal Medium Eagle (BME) with 10% calf serum and the pH of the cultures was buffered at 7.2 and 7.4. Coverslips grown in culture tubes were used for staining with oil red O and hematoxylin. For studies of total cell protein by a modification of the Lowry method Falcon T-25 flasks were used. Due to the very low solubility of both DEHP and BGBP, these compounds were first stirred into the calf serum moiety of the medium for 22 hours. The resulting serum emulsion was added to the BME medium and stirred for another 22 hours. Concentrations of 0.25 mM, 0.50 mM, and 1.00 mM were prepared. It should be pointed out, however, that these amounts do not represent the actual concentration (see below), because both compounds were incompletely solubilized.

Di-2 ethylhexyl phthalate

Preliminary staining revealed a noticeable growth inhibition with 1.0 mM DEHP added to the medium. After 6 days, the treated cell population is less than one half the density of the controls. The cells show a stretched appearance, although the nuclei appear normal.

A more detailed study of the effects of DEHP is shown by protein determination in fig. 5. Each point represents the mean

value of three sample T-25 flasks. Concentrations of 0.25 mM, 0.50 mM, and 1.00 mM DEHP were studied. To find the actual amount of DEHP solubilized in the medium, samples were centrifuged twice to separate the DEHP emulsion droplets from the clear medium. The clear fraction was extracted and quantitated by GLC. The values found for 0.25 mM, 0.50 mM, and 1.00 mM were 0.051 mM, 0.069 mM, and 0.160 mM respectively. These figures indicate that approximately 15-20% of the DEHP added was solubilized. The T-25 flasks treated with 0.25 mM and 0.50 mM DEHP showed decreased cell growth, although the cells were still viable at the end of this experiment. The flasks treated with 1.00 mM DEHP exhibited cell degeneration by day 7, which subsequently resulted with death of the population. From these data, the ID_{50} (dose required to inhibit cell growth by 50%) is estimated at 0.07 mM DEHP for WI-38 cells.

A heavy cell population with 1.6 mg. protein per flask on day 4 (fig. 6) was treated with both DEHP and BGBP for 9 days. In general, replication occurred much more slowly than in a lower density population (see fig. 5). Using 0.50 mM DEHP, the cell population showed an initial growth increase, followed by a stabilization of protein near the original value. In terms of absolute protein, the 0.50 mM DEHP flask of the slower growing population contained 59% of the control protein value after 9 days of treatment. In comparison, the rapidly replicating population of fig. 5 demonstrates that 0.50 mM DEHP flasks contains only 50% of the protein contained in the controls; a 10% difference. The data suggests that replicating cells are more strongly affected by DEHP.

Other experiments were conducted to test the reversibility of the effects of DEHP. Cells were grown in the presence of 0.50 mM and 1.00 mM DEHP for either 3 or 5 days, and then subcultured and planted at equal viable cell densities in control medium. There was an approximate 50% decrease in the regrowth of cells treated with 1.00 mM DEHP for 3 days, and a 75% decrease in the regrowth of those cells treated for 5 days before subculture.

Butyl carbobutoxymethyl phthalate

Staining cells treated with BGBP demonstrated effects similar to those shown with DEHP. A concentration of 1.00 mM BCBP added to the medium results in a decreased cell density. These cells appeared stretched, with more cytoplasmic thinning than is observed with DEHP.

Detailed protein determination for various concentrations of BCBP is now being conducted. It appears that 0.25 mM, 0.50 mM, and 1.00 mM BGBP produce a curve similar to that demonstrated by similar concentrations of DEHP in the medium. A GLC analysis of the actual amount of BCBP in solution is currently being conducted.

Available data reveals the effects of 0.50 mM and 1.00 mM BGBP on a heavy cell population (fig. 6), namely, a noticeable decrease in total cell protein at these concentrations.

Cells grown in the presence of 0.50 mM and 1.00 mM BGBP, and subsequently subcultured into control medium did not deviate from control values.

The findings to date indicate that both DEHP and BGBP have measurable effects on WI-38 cells, a diploid human cell. Further

experiments, using ^{14}C DEHP and ^{3}H DEHP (or a combination of both), will allow us to determine intracellular DEHP localization by autoradiography and cell fractionation techniques. These projected studies will allow us to determine whether DEHP is accumulated and stored in WI-38 cells, or eliminated and/or metabolized over time.

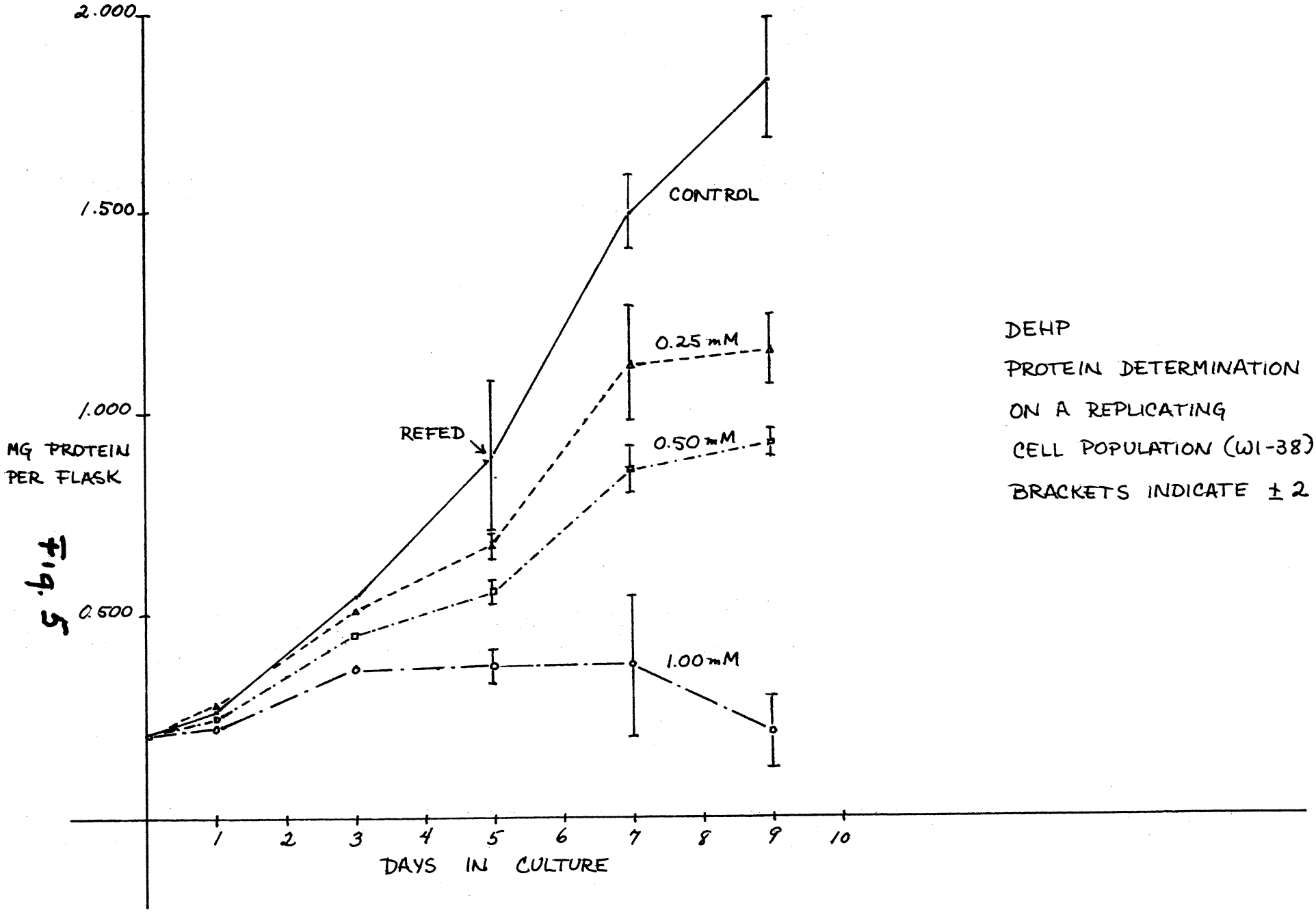


Fig. 5

DEHP
PROTEIN DETERMINATION
ON A REPLICATING
CELL POPULATION (WI-38)
BRACKETS INDICATE ± 2 STD. ERR

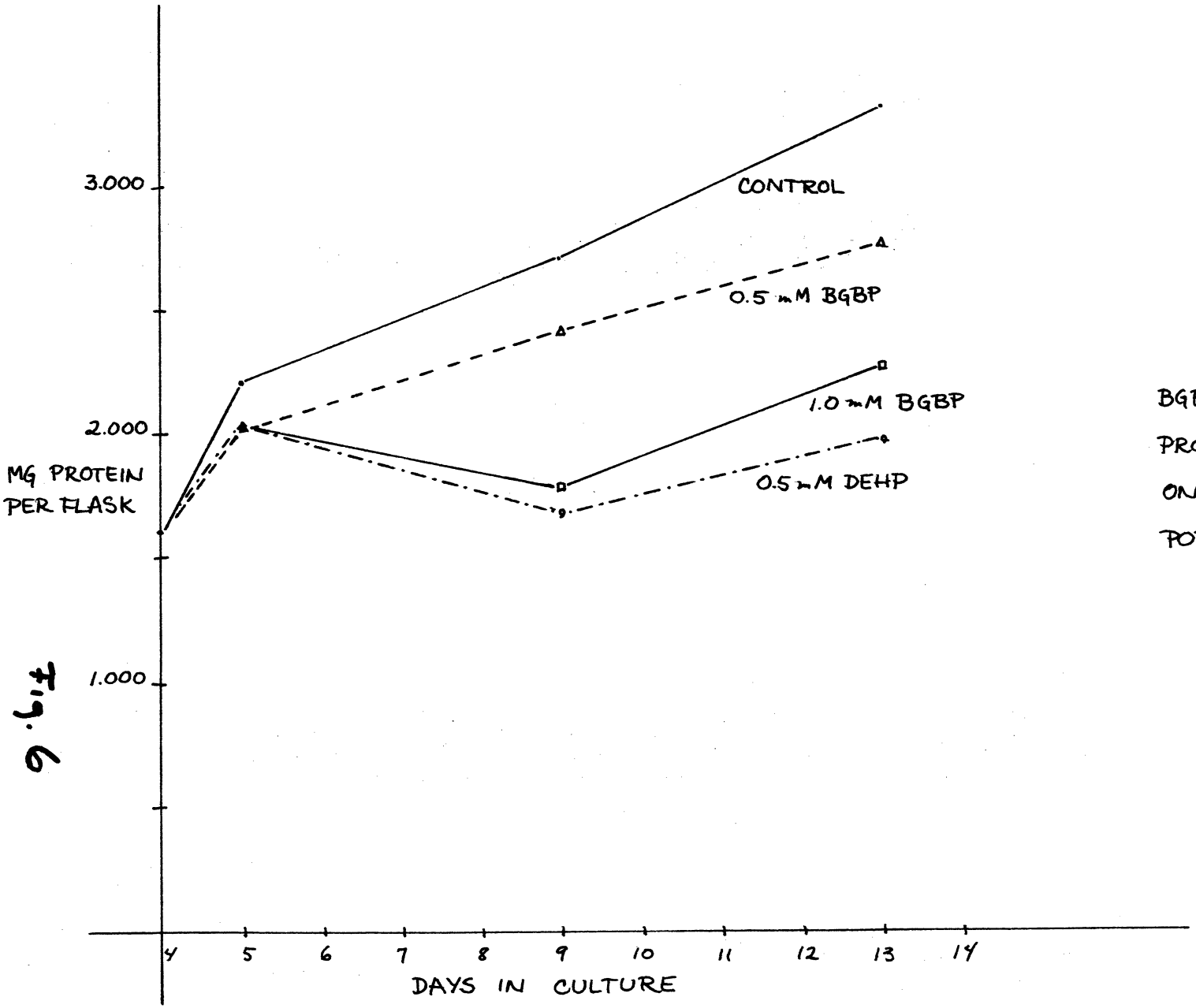


Fig. 6

BGBP & DEHP PROTEIN DETERMINATION ON A HEAVY CELL POPULATION. (WI-38)

Effects of DEHP on Blood Coagulation in vitro

DEHP was added to normal human plasma to evaluate the effects of this agent on blood coagulation. The following studies were obtained: platelet aggregation, clot retraction, prothrombin time, partial thromboplastin time, thrombin clotting time, euglobulin whole blood lysis time. Specific factor asctivities also were measured which included fibrinogen, Factor VIII, IX, X, XI and XII.

Procedure: DEHP in concentrations of 0.01mM and 0.1mM was added to platelet poor and platelet rich plasma directly with active mixing and in a methanol solution. A methanol blank was employed as a control for the methanol solution. The values for clot retraction and plasma coagulation assays are illustrated in Table I. The control (or blank) was considered as a 100% activity for the factor assays VIII, IX, X, XI, XII.

Platelet aggregation studies were undertaken with the addition of adenosine diphosphate to provide a final concentration in platelet rich plasma of .25 and 1 microgram per ml, epinephrine .025 mg/ml and collagen 0.1 mg/ml.

The addition of DEHP either at the 0.1 or 0.01 mM did not appear to affect the clotting parameters studied as indicated by the lack of significant differences between the control and DEHP values (results are within the range of error for each procedure). We would conclude that exposure of normal human plasma and platelets to DEHP does not affect the coagulation mechanism.

DEHP IN VITRO STUDIES TO DETERMINE THE EFFECTS ON COAGULATION

Normal human platelet free and platelet rich plasma were prepared, mixed with DEHP directly or in methanol and incubated for 30 min. at 37°C.

DEHP in Methanol:

	C.R. %	P.T. sec	P.T.T. sec	T.C.T. sec	Fi mg%	W.B. Eugl. % lysis	Factor VIII %	Factor IX %	Factor X %	Factor XI %	Factor XII %
Blank	90	10.9	35.8	7.8	220	10	100	100	100	100	100
0.1mM	90	10.8	34.7	7.2	205	15	156	110	108	99	93
0.01mM	90	10.8	34.6	7.4	205	15	108	106	123	122	105
DEHP in plasma:											
Control	80	11.0	40.6	7.8	220	10	100	100	100	100	100
0.1mM	85	11.1	41.5	7.6	220	10	105	88	106	84	99
0.01mM	90	11.0	40.2	7.8	220	10	103	72	115	92	104

PROTOCOL: Obtaining Autopsy Tissue for Plasticizer Study

In order to determine tissue levels of DEHP and any other plasticizer deemed important, adequate samples of seven tissues will be obtained during consecutive autopsies performed at University Hospital Ann Arbor, Michigan.

Tissues sampled will be: (1) kidney (2) spleen (3) liver (4) heart (5) omental fat (6) lung and (7) brain. If possible, 50 grams of each tissue will be obtained. In the case of adults, this amount of tissue will be readily available from kidney, spleen and liver. Since the prosector normally saves only one-half of each kidney, the discarded tissue generally will amount to over 100 grams. In sampling the brain, the anterior tip of one or both frontal lobes can be saved. In sampling the heart, care must be taken not to disrupt the appearance of the anatomic specimen if it is to be displayed. Adequate heart muscle can be obtained by taking a strip of tissue from the wall or walls of the left ventricle parallel to the septum after the prosector has opened the heart. In sampling lung, it must be remembered that the weight of this organ can be substantially increased by the presence of edema. If fluid is noted to be exuding from the lung parenchyma, approximately double the desired amount of lung by weight should be obtained.

Fifty grams of each tissue would be optimal. Ten grams or even less generally will be sufficient to do the assay. In the case of pediatric patients or newborn infants where the amount of tissue is limited, as much tissue as possible should be obtained.

All tissue will be obtained fresh before any contact with fixatives. The tissue obtained will be as fresh as possible. The time interval between death and autopsy is quite variable and governed by factors beyond the control of the prosector.

A log book will be maintained and all data carefully recorded by the individual obtaining the tissues. For each autopsy, the following will be noted: (1) the patient's name, sex and age (2) autopsy number (3) name of prosector (4) date of autopsy (5) tissues obtained and amount in grams and (6) brief clinical history including transfusion history, if available.

Tissues will be obtained from autopsies performed on weekdays. Special effort will be made to obtain tissue from patients generally free of organic disease, such as car accident victims. No additional special effort will be made to select patients and tissue will be obtained from as great a percentage of routine autopsies as possible.

SYNTHESES OF LABELLED COMPOUNDS

1. Bis(2-ethylhexyl)phthalate-$\underline{d}_4$

Attempts to effect isotopic exhange directly on commerically available bis(2-ethylhexyl)phthalate under a variety of conditions failed to give appreciable deuterium incorporation, however, the following scheme allowed efficient preparation of DEHP-$\underline{d}_4$ with the protons of the aromatic ring fully deuterated.

Phthalic acid was converted to its potassium salt and treated with platinum dioxide-deuterium gas in D_2O solvent. The mixture was heated on a Paar shaker for 48 hr. After three successive treatments under the conditions above phthalic acid-$\underline{d}_4$ was isolated by acidification. The extent of deuterium incorporation was determined to be greater than 95% by nmr and mass spectrometry.

Phthalic acid-$\underline{d}_4$ (0.5 g, 0.00294 mole) was dissolved in 4 ml 2-ethylhexanol and added to 75 ml toluene containing three drops conc. sulfuric acid. The mixture was refluxed in a flask fitted with a Dean-Stark trap for azeotropic removal of water. After 24 hr the reaction mixture was taken up in 200 ml ether and extracted with aq. sodium bicarbonate. Toluene and excess 2-ethylhexanol were removed under vacuum (160°/0.1 mm) after removal of the ether. The crude product was chromatographed on Woelm silica gel with benzene-ethyl acetate (95:5) to afford DEHP-$\underline{d}_4$ (1.0 g, 90% yield). The structure and isotopic composition of the product was determined by mass spectrometry and nmr.

2. Bis(2-ethylhexyl)phthalate-t

2-Ethylhexanol (2.6 g, 0.02 mole, 10 mCi-tritium) and phthaloyl chloride (2.00 g, 0.00985 mole) were dissolved in 50 ml tetrahydrofurance containing

pyridine (1.58 g, 0.02 mole). The mixture was stirred at room temperature for 12 hr, filtered to remove pyridine hydrochloride, and the tetrahydrofurane evaporated. The crude product was column chromatographed on silica as above to afford DEHP-t (2.8 g, 75% yield). This material was homogeneous to glpc.

3. Bis(2-ethylhexylphthalate-^{14}C

Phthalic anhydride (2.5 g, 0.017 mole, 10 mCi-carbonyl-^{14}C) and 2-ethylhexanol (5 ml) were dissolved in 10 ml toluene containing four drops conc. sulfuric acid. The mixture was refluxed for 5 days with azeotropic removal of water. The reaction mixture was taken up in 200 ml ether with aq. sodium bicarbonate. The organic layer was dried and concentrated under vacuum. Excess 2-ethylhexanol was distilled away (160°/0.01 mm). Attempted distillation of the product led to decomposition above 210°. The product was purified by chromatography on silica gel as above (6.0 g, 90% yield).

4. Quantitative Analysis of DEHP at Sub-nanogram Levels

The scope of the proposed research required the development of a rapid, sensitive and discriminating analytical method for the presence of phthalate esters possibly as only trace constituents of complicated mixtures. Given these constraints we have developed a gas chromatograph-mass spectrometric technique which allows quantitative detection of DEHP in sub-nanogram levels even in the presence of contaminating materials that may not be separated from DEHP by gas chromatography alone. The unique capabilities of a Multi-peak Monitor accessory on a computerized mass spectrometer make this technique possible. Most phthalate esters have a highly characteristic base ion in the mass spectrum at m/e = 149 corresponding to $C_8H_5O_3^+$-protonated phthalic anhydride.

Accordingly, the base peak in the mass spectrum of DEHP-$\underline{d}_4$ is found at m/e = 153 ($C_8HD_4O_3^+$) and in mixtures of DEHP and DEHP-$\underline{d}_4$ we have found the ratio of ion current at m/e = 149 and m/e = 153 to be an accurate measure of the molar ratio of these two substances. Thus, addition of a known quantity of DEHP-$\underline{d}_4$ to a sample containing umknown amount DEHP and measurement of the m/el49-m/el53 ion current ratio at the point corresponding to the retention time of DEHP in the GC-mass spectrum allows a determination of the amount of DEHP in the sample. Given the extreme sensitivity of the mass spectrometer, operating in this case as a detector for a gas chromatograph and the ability of this technique to overcome uncertainties due to the presence of other materials, we are not able to routinely assay for DEHP and other phthalates at very low levels with high accuracy and reliability. This newly developed capability will be invaluable in the upcoming phases of the program (cf. Tables II, III, and Figure 7).

Table II

Quantitative DEHP/DEHP-d_4 Values

Solutions Prepared in Acetone in 10-ml Volumetric Flasks		Volume Ratio, DEHP/DEHP-d_4	Peak Ratio, 149/153
DEHP	DEHP-d_4		
1 µl	--		149 1.5 - 1.6 cm
5 µl	1 µl	5.0	5.526, 5.808, 2.38
2 µl	1 µl	2.0	2.33, 2.1, 2.34, 1.167
1 µl	1 µl	1.0	1.0, 1.136, 1.156
1 µl	2 µl	0.5	0.583, 0.457, 0.553
1 µl	5 µl	0.2	0.234, 0.229
--	1 µl		1.5, 1.45 cm

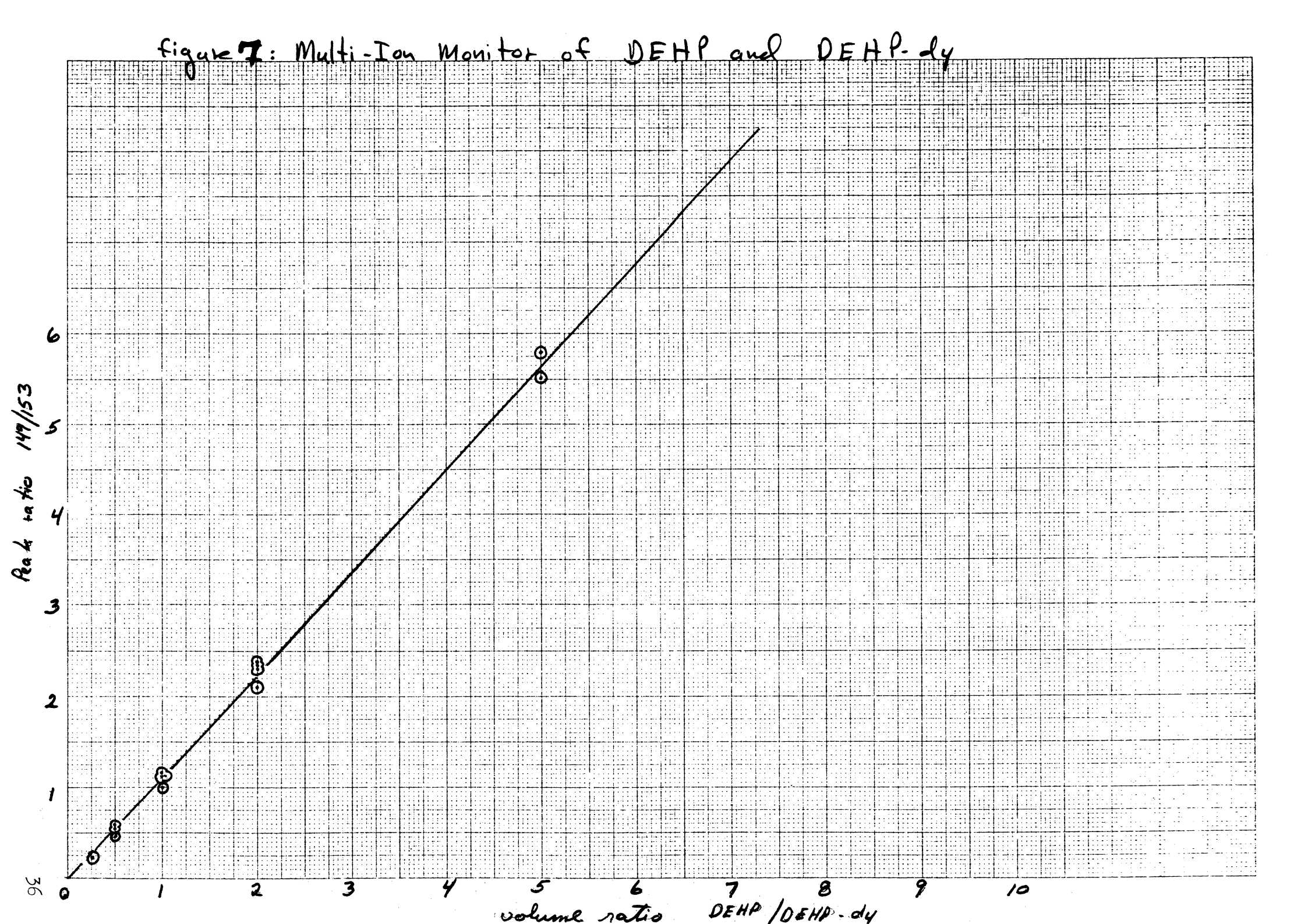
Table III

Limit of Detection of m/e149 of DEHP on Multi-peak Monitor

Run No.	1 µl Injection of	Grams	Sensitivity	Range	eV	Peak Height
1	1 µl DEHP + 1 ml acetone	10^{-6}	6.0	X30	20	Off scale
2	1 µl DEHP in 10 ml acetone	10^{-7}	6.0	X30	20	1 cm
3	Pure acetone (blank)	--	7.0	X 3	20	No peak
4	1 µl DEHP in 100 ml acetone	10^{-8}	7.0	X 3	20	0.7 cm
5	1 µl DEHP in 100 ml acetone	10^{-8}	7.5	X 1	20	1.2 cm
6	1 µl DEHP in 100 ml acetone	10^{-8}	9.0	X 1	20	Off scale
7	1 µl DEHP in 100 ml acetone	10^{-8}	9.0	X 3	20	Off scale
8	1 ml ④ diluted to 10 ml	10^{-9}	9.0	X 3	20	0.9 cm
9	1 ml ④ diluted to 10 ml	10^{-9}	9.5	X 3	20	0.7 cm
10	1 ml ④ diluted to 10 ml	10^{-9}	9.5	X 3	70	1 cm
11	1 ml ④ diluted to 10 ml	10^{-9}	9.5	X 3	70	? Off peak

Samples were run in 1% SE30 at nominal 220 C. DEHP had a retention time of ~6.5 min. The detection limit will vary with the retention time of the compound and also with the condition of the source. Just after source cleaning, the detection limit should be at its best.

figure 7: Multi-Ion Monitor of DEHP and DEHP-d4



EXTRACTION OF DEHP FROM BIOMATERIALS USED IN HEMODIALYSIS

Plastic tubing for hemodialysis from two commercial sources was primed with two liters of saline and then extracted with 500 to 700 ml of human plasma recirculated through the tubing at 37°C at 200 ml/min. The plasma was obtained as a by-product of packed red blood cell preparation in the blood bank and was in a plastic container less than 30 min before use. Results are given in Table IV. The low levels of DEHP in the plasma before extraction probably came from the brief exposure to the plastic systems used in the separation process. DEHP concentration in the plasma rose progressively during both experiments. The rate of increase was markedly reduced when the recirculation was discontinued during the second hour of experiment A.

We conclude that (a) the peak plasma levels achieved are approximately tenfold greater than previously reported for a similar blood tubing system plus the hemodialyzer, and (b) the concentration of DEHP was tenfold greater than that known for toxic concentration for cells and tissue culture. Studies are in progress to extend these observations, to determine the extraction of DEHP from hemodialyzers per se which are profused by the blood tubing tested thus far, and to estimate the accumulation of DEHP in patients who require hemodialysis.

Table IV

Accumulation of DEHP in Human Plasma Perfused Through Two Different Commercial Hemodialysis Units

Hourly Samples Removed from Unit	Micrograms of DEHP per Milliliter Perfused Human Plasma
Unit A: Control	2.95
1st hr	5.59
2nd hr	5.65
3rd hr	10.67
4th hr	13.60
5th hr	15.47
Unit B: Control	1.13
1st hr	6.20
2 hr	10.77
3rd hr	16.50
4th hr	18.48
5th hr	28.90
6th hr	27.52

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15. Supplementary Notes

16. Abstracts

Phthalate esters, commonly used as plasticizers, are ubiquitous in their occurrence; but their effect on human health has yet to be ascertained. For studies of the metabolism of phthalates in human cell cultures, double-labeled radioactive phthalates (^{14}C; ^{3}H) have been synthesized, and in vitro studies are now in progress. Correspondingly, double-labeled stable isotope esters have also been synthesized (^{13}C; ^{2}H), which will be utilized to study their metabolism in vivo by humans. Our laboratories have shown, in vitro, that phthalates are readily leached out into solutions perfused through various plastic hemodialysis units, even if only saline is used as the perfusing solution. With lipid containing solutions, such as human plasma or corn oil, correspondingly considerably more phthalate esters are in these solutions subsequent to perfusion. Remarkably, however, in the assay of patients plasma subsequent to hemodialysis using the same units (normally for a period of 6 hr), phthalates were very difficult to detect in vivo, suggesting that tissues are rapidaly clearing the plasma of plasticizers. Toxicity studies with human cell cultures (WI-38 fetal diploid, fibroblasts, 50 generation ± 10) have shown a clear fatal dose-response relationship, at phthalate concentrations of 0.01 to 1.0 mM over a period of several days exposure of the cells. In our preliminary monitoring studies of human tissues, phthalates have been detected in hearts, liver, lung, spleen and kidney; our data, however, are too preliminary at the present date to correlate the occurrence of the phthalates with patient histories.

17. Key Words and Document Analysis. 17a. Descriptors

Plasticizers; double-labeled radioactive phthalates; double-labeled stable isotope esters; metabolism in vivo by humans; hemodialysis units; toxicity; human cell cultures

17b. Identifiers/Open-Ended Terms

17c. COSATI Field/Group

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