THE UNIVERSITY OF MICHIGAN

MEDICAL SCHOOL
Departments of Biological Chemistry and Internal Medicine

Progress Report

ACCUMULATION, TOXICITY, AND METABOLISM OF COMMON PLASTICIZERS IN HUMANS

Period covered: December 1972 - May 1974

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 project.

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.

In addition to the above Principal Investigators, considerable assistance has been received by the participation of Dr. Bruce A. Friedman, Assistant Professor of Pathology.

SUMMARY

Synthesis of di-2-ethyl-hexyl phthalate (DEHP) double-labeled with both stable isotopes (carbon-13 and deuterium) and radioactive isotopes (carbon-14 and tritium), has been completed, and also distributed to other contractors at their request. The synthetic procedures employed for obtaining the various double-labeled DEHP isomers, important for both our in vitro and in vivo studies, are now being extended to the other phthalates as outlined in our original experimental design.

In our in vitro coagulation studies with human platelets, similar results for both di-2-ethyl-hexyl phthalate (DEHP) and butyl carbo-butoxy-methyl phthalate (BCBP) were obtained when these compounds were incubated with human platelets at 37°C for a period of 30 min. Somewhat inconclusive results were obtained, and these "short period" studies will be repeated. Further coagulation studies with platelets isolated from whole blood and plasma that have been stored in the blood bank for various times (up to 21 days) are now being conducted. That is, platelets will be isolated (e.g., every 3 days) from these tissues stored in the PVC bags, to note any "long term" effect of phthalates on platelets with respect to coagulation mechanisms.

In our monitoring program for the occurrence of DEHP in human tissues obtained at necropsy, we have found measurable levels of DEHP in 27 of 116 tissues analyzed to date (ca., 23%). With our current preliminary data, DEHP was detectable in 5 of 23 livers, 11 of 23 spleens, 1 of 19 hearts, 4 of 20 lungs and, 2 of 21 kidneys. It should be emphasized that these are very

limited preliminary data which do not permit us to make correlations with medical histories (for example, we have data from only 5 patients who have had a history of long-term hemodialyses).

An important part of our program, not detailed in this report, is our continuing studies of the binding of DEHP to various human plasma proteins other than albumin (cf., Annual Progress Report, October 1973). Recently, through the courtesy of Dr. James Sgouris of the State of Michigan Health Laboratories, we have received proteins (albumin, the various globulins, fibrinogin, etc.) fractionated from 700 liters of human plasma by the Cohn technique. In this regard we hope to obtain information of the distribution of DEHP that was in the initial plasma.

Organic compounds found leachable from plastic medical devices, in addition to phthalates, were identified by mass spectrometry; and these data were presented before the 1974 midwestern spring meeting of the American Chemical Society. The abstract of this presentation appears as Addendum II.

The quantitation of the leakage of plasticizers into human plasma circulated in vitro through various commercial hemodialysis units for periods of up to 6 hours, has been completed for five units; Addendum III, a summary of these data, is a copy of a manuscript accepted for publication in Proceedings of the Society for Experimental Biology and Medicine.

Finally, data on in vitro toxicity of the plasticizers on human cell cultures have been summarized into a manuscript that was submitted for publication in the journal Toxicology of Applied Pharmacology; a copy of the submitted manuscript appears as Addendum IV.

SYNTHESES OF LABELED COMPOUNDS

1. BIS(2-ETHYL-HEXYL) PHTHALATE-$\underline{D}_4$; LABELED SIDE CHAIN

DEHP with four deuterium atoms in the aromatic ring (DEHP-(Ar)$\underline{d}_4$), with carbon-14 at the acyl position (DEHP(Ac)-^{14}C) and with tritium in the alcohol side chain (DEHP-(R)$\underline{t}$) have been synthesized as described in the Annual Report (No. NHLI-73-2936-B). Larger quantities of these materials needed by other contractors as well as ourselves have been prepared. Since phthalate ester metabolism has been shown to involve side chain hydrolysis and subsequent ω-oxidation of the resultant half-ester, the incorporation of stable isotopes into the side chain of DEHP was required for the <u>in vivo</u> studies in humans. 2-Ethyl hexanol with two deuterium atoms in the α-position was synthesized as outlined in the renewal proposal (May 1974). Accordingly, commercial 2-ethyl hexanol was oxidized with chromic anhydride in pyridine to afford 2-ethyl hexanoic acid. The acid was converted to its methyl ester by treatment with HCl/methanol and the ester purified by distillation. Reduction of the methyl 2-ethyl hexanoate with lithium aluminum deuteride in ether at room temperature and quenching of the reaction mixture with water gave crude 2-ethyl hexanol-α-$\underline{d}_2$ which was purified by distillation under vacuum. The retention time of the product by glpc (3% SE-30) was identical to that of starting material and it had accordant spectroscopic properties.

The 2-ethyl hexanol-α-$\underline{d}_2$ was converted to the correspondingly labeled bis(2-ethyl-hexyl) phthalate-$\underline{d}_4$ (DEHP-(R)$\underline{d}_4$) by reaction with a 0.5 molar equivalent of phthaloyl chloride in tetrahydrofurane containing one equiva-

lent of pyridine. The mixture was stirred at room temperature for 12 hr, filtered to remove pyridine hydrochloride, and the solvent was evaporated. The crude product was treated with activated charcoal and column chromatographed on Woelm silica gel with benzene-ethyl acetate(95:5) to afford the product DEHP-(R)$\underline{d}_4$ in 75% yield. The product was clear, colorless and homogeneous to glpc upon coinj tion with an unlabeled sample.

2. PURIFICATION OF BUTYL CARBO-BUTOXY-METHYL PHTHALATE

Commercial samples of butyl carbo-butoxy-methyl phthalate were found to contain an impurity in amounts as high as 10-15% (Annual Report No. NHLI-73-2936-B, p. 16). This impurity has now been identified as dibutyl phthalate by G. C.-mass spectrometry. Since initial studies by us have shown high cellular toxicity of dibutyl phthalate, any results obtained on impure butyl carbo-butoxy-methyl phthalate would be inconclusive. Accordingly, we have sought, as with all other compounds under investigation, to prepare rigorously pure samples. These efforts have been made more difficult by several factors; the method of synthesis (Renewal Proposal, November 1973, p. 8) virtually guarantees contamination with dibutyl phthalate and transesterification of apparently pure butyl carbo-butoxy-methyl phthalate to afford dibutyl phthalate occurs upon standing (probably $\underline{\text{via}}$ surface reaction with the glass. While our standard column chromatographic techniques did not cleanly separate these compounds, pure BCP was finally obtained by multiple elution on silica gel and careful monitoring of the eluate by glpc (3% SE-30 at 210°).

3. BIS(2-ETHYL-HEXYL) PHTHALATE-$\underline{D}_4$

Attempts to effect isotopic exchange directly on commercially available bis(2-ethyl-hexyl) 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-ethyl hexanol 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-ethyl hexanol 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.

4. BIS(2-ETHYL-HEXYL) PHTHALATE-T

2-Ethyl hexanol (2.6 g, 0.02 mole, 10 mCi-tritium and phthaloyl chloride

(2.00 g, 0.00985 mole) were dissolved in 50 ml tetrahydrofurane 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.

5. BIS(2-ETHYL-HEXYL) PHTHALATE-^{14}C

Phthalic anhydride (2.5 g, 0.017 mole, 10 mCi-carbonyl-^{14}C) and 2-ethyl hexanol (5ml) 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-ethyl hexanol 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).

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.

The base peak in the mass spectrum of DEHP-d_4 is found at m/e = 153 ($C_8HD_4O_3^+$) and in mixtures of DEHP and DEHP-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-d_4 to a sample containing unknown amount DEHP and measurement of the m/e149-m/e153 ion current ratio at the point corresponding to the retention time of DEHP in the G.C.-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 now 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 I, II, and Figure 1).

TABLE I

QUANTITATIVE DEHP/DEHP-$\underline{d}_4$ VALUES

Solutions Prepared in Acetone in 10-ml Volumetric Flasks		Volume Ratio, DEHP/DEHP-$\underline{d}_4$	Peak Ratio, 149/153
DEHP	DEHP-$\underline{d}_4$		
1 µl	--		149 1.5 - 1.6 cm
5 µl	1 µl	5.0	5.526, 5.808
2 µl	1 µl	2.0	2.33, 2.1, 2.34
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 II

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.

EFFECTS OF DEHP AND BCBP ON BLOOD COAGULATION IN VITRO

Phthalate 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 activities also were measured which included fibrinogen, Factor VIII, IX, X, XI, and XII.

Procedure: Phthalate in concentrations of 0.01 mM and 0.1 mM 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 Tables III and IV. 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 μg/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 (Table III).

The addition of BCBP either at the 0.1 or 0.01 mM level did not appear to affect the clotting parameters studied with the exception of the platelet

TABLE III

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.

	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 %
DEHP in Methanol:											
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

TABLE IV

BUTYL CARBO-BUTOXY-METHYL PHTHALATE (BCBP)

	C.R. %	P.T. sec	P.T.T. sec	T.C.T. sec	Fi mg%	Eugl. % Lysis	Factor VIII %	IX %	X %	XI %	XII %
Blank with Methanol	90	11.2	37.2	7.9	185	10	100	100	100	100	100
0.1mM with methanol (final conc. in plasma)	90	11.0	32.4	7.9	185	10	94	90	124	109	106
0.01mM with methanol	90	11.0	35.9	7.9	185	10	77	77	124	148	82
Blank	85	11.0	36.0	7.8	185	10	100	100	100	100	100
0.1mM BCBP without methanol dir. in plasma	80	11.0	33.9	8.4	190	10	95	73	113	114	107
0.01mM BCBP	80	11.0	36.4	8.3	185	10	73	97	96	115	93

studies. No significant difference was observed between the control values using a methanol or plasma blank and the samples containing the plasticizer (Table IV).

Platelet aggregation studies with adenosine diphosphate and collagen failed to demonstrate an effect from the plasticizer; however, the absence of the epinephrine induced secondary wave was noted when the plasticizer was incubated with the normal plasma. The secondary wave also was absent when the plasticizer was added in methanol, however, the blank for this experiment (methanol) also failed to develop a secondary wave. These results would suggest that the platelets are affected by concentrations of the plasticizers at a level of 0.01 mM and would be similiar to the effect seen with some aspirin containing compounds. The study will have to be repeated for confirmation. It should be noted that the methanol alone has produced similar abnormalities on several occasions and may be responsible for the abnormal results seen in this portion of the study. The plasticizers' solubility in plasma is poor and the possibility that particulate matter is responsible for the abnormal results seen in this aspect of the study also should be considered (Table IV).

The effect of plasticizers on platelets and coagulation after the storage of blood or plasma for varying periods of time, at which time intervals platelets have been separated, is not clear however; and these studies are now in progress. Specifically, plasticizers will be added to whole blood and plasma immediately after collection ("zero time"), and platelets will be isolated from these stored tissues after varying time intervals (e.g., every three days), up to the normal storage time of 21 days, and coagulation studies will be

undertaken. Platelets isolated from the same stored whole blood and plasma, without added plasticizers at "zero time," will similarly be assayed as control studies on the coagulation mechanism effects.

MONITORING OF HUMAN TISSUES FOR THE PRESENCE OF PLASTICIZERS

From July 1973 until the present, various tissues have been obtained from 41 necropsies performed at University Hospital, Ann Arbor, Michigan. A special attempt has been made to obtain tissue from patients who have undergone chronic renal dialysis and also from those who have received multiple transfusions of blood and blood products.

From each necropsy, an attempt was made to obtain a sample of heart, lung, kidney, liver, spleen, brain, and omental fat. This goal could not always be accomplished due to factors such as limited autopsy permits.

To date, 194 separate tissue specimens have been obtained. Of this number 116 have been analyzed for DEHP. Nearly all of the remainder have been completely processed and are awaiting analysis. Both omental fat and brain have proven difficult to analyze for technical reasons (which have been resolved).

RESULTS TO DATE

Refer to Table V for the quantitative tissue assay data for DEHP.

TENTATIVE CONCLUSIONS UTILIIZING AVAILABLE DATA

(1) The liver appears to frequently contain significant quantities of DEHP. Since analysis of plasma has consistently shown that DEHP is rapidly cleared from it in vivo, one may surmise that the liver, an integral part of the reticuloendothelial system, participates in this effect. These tissue data, however, would seem to contradict those of Jaeger and Rubin (New Engl. J. Med. 287:1114, 1972) who found large amounts of DEHP in lung, particularly in cardiopulmonary bypass patients.

(2) The spleen frequently contains DEHP. This finding may reflect the participation of the organ in the reticuloendothelial system.

TABLE V*

μg DEHP/GRAM WET TISSUE

Necropsy No.	Age & Sex	Clinical Diagnosis	Transfusion and Hemodialysis History	Liver	Omentum	Spleen	Heart	Lung	Kidney	Brain
A-15	44 M	90% whole body burn	7 units whole blood, 10 units plasma	0	TBA	0	0	0	NA	NA
A-16	50 M	Acute rejection cadaver renal transplant	5 units whole blood, 2 years hemodialysis three weekly	0	TBA	0	0	0	0	NA
A-23	41 M	Acute myocardial infarction	None known	NA	0	0	0	0	0	0
A-52	40 F	Chronic granulocytic leukemia	16 units platelets, 3 units RBC's	0	TBA	.0223	0	.0205	0	NA
A-53	64 M	Bronchogenic carcinoma	None known	0	TBA	0	0	0	0	NA
A-61	4wkM	Multiple congenital anomalies	20 ml RBC's	.008	NA	.0062	NA	NA	NA	NA
YA-89	90 F	Arteriosclerotic heart disease	None known	0	0	0	0	0	0	NA
A-90	25 F	Gram negative shock post-partum	22 units platelets, 23 units RBC's, 6 units plasma, 3 units blood	0	TBA	TRACE	NA	NA	0	NA
A-92	2day M	Potter's syndrome with 69 chromosomes	None Known	0	NA	NA	NA	NA	NA	.008
A-98	53 F	Idiopathic cardiomyopathy	None known	0	NA	0	0	TBA	0	NA
A-107	26 M	Wolf-Parkinson-White syndrome	None known, plasmaphoresis donor for 1 year	0	TBA	0	NA	0	0	.0228
A-163	53 M	Chronic granulocytic leukemia	9 units RBC's 5 units platelets	0	TBA	TRACE	0	TRACE	0	TBA
A-164	52 M	Bronchogenic carcinoma	None known	0	TBA	0	0	0	0	TRACE
A-192	20 M	Carbon monoxide poisoning in housefire	None known	0	0	.034	0	0	0	NA

TABLE V (Concluded)

Necropsy No.	Age & Sex	Clinical Diagnosis	Transfusion and Hemodialysis History	Liver	Omentum	Spleen	Heart	Lung	Kidney	Brain
A-200	66 F	Mital valve disease with insertion of valve prosthesis	26 units whole blood	.0323	0	.0310	NA	.0086	.0065	NA
A-201	38 M	Acute myelo-monocytic leukemia	7 units whole blood, 14 units RBC's, 6 units platelets, 5 units plasma	0	TBA	0	0	0	0	TBA
A-202	59 M	Acute myelo-monocytic leukemia	None known	0	TBA	NA	TRACE	0	0	NA
A-203	50 M	Glioblastoma	2 units whole blood 5 months previously	0	TBA	0	0	0	.2538	NA
A-213	38 M	Acute rejection cadaver renal transplant	Hemodialysis history unknown	TBA	TBA	.0522	0	0	NA	.044
A-223	30 F	Chronic granulocytic leukemia, hypersplenism	10 units whole blood, 2 units leukocyte-poor RBC's	.0158	TBA	.550	0	NA	0	NA
A-235	53 M	Acute myelo-monocytic leukemia, pancytopenia	31 units platelets 13 units RBC's	0	TBA	0	NA	0	0	NA
A-236	59 M	Carcinoma of head of pancreas	12 units platelets, 16 units whole blood, 28 units packed cells, 3 units plasma, hemodialysis 7x	0	TBA	0	0	0	0	TBA
A-249	50 F	Anephric, history of chronic renal failure	4 units packed cells, 3 units plasma, hemodialysis 3x/wk (duration not known)	TBA	TBA	TRACE	0	.0094	NA	TBA
A-277	5day M	Erythroblastosis fetalis due to Rh incompatibility	8 units packed cells, 7 units plasma, 3 units platelets	.0141	TBA	NA	NA	NA	NA	NA
A-303	28 M	3 cadaverine renal transplants (3 yrs ea.)	Hemodialysis approx. 9 years	.0112	NA	.0245	NA	NA	0	NA
A-318	22 M	Bilateral nephrectomy & donor renal transplant	Hemodialysis 3x/wk for 10 months	TBA	TBA	NA	0	0	0	0
A-352	61 M	Motor vehicle accident	None known	TBA	TBA	.0139	0	NA	0	TBA
A-355	45 F	Congestive heart failure, systematic lupus erythromatosis	None known	0	TBA	TBA	TBA	0	TBA	TBA

*TBA - To be analyzed; NA - Tissue not available; TRACE - Detectable trace which could not be calculated

(3) Surprisingly, only one out of 19 heart specimens analyzed contained DEHP (particularly since DEHP was consistently found in hearts analzed from other species (Biochem. 10:4228, 1971).

(4) Of 20 lung specimens analyzed 16 did not contain detectable DEHP and the remainder contained low amounts of the chemical.

(5) The greatest single concentration of DEHP was found in the spleen of one patient with hypersplenism, with a known recent history of transfusion (A-223).

(6) Of the five patients who underwent chronic renal dialysis with the attendant high exposure to plastic material, three had detectable amounts of DEHP.

The above preliminary notations are tentative due to limited data. Final more definitive correlation with medical histories must await further tissue analysis obtained from significantly more necropsies.

ADDENDUM I

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

Phthalate	Retention Time
Dimethyl	0.26 minutes
Diethyl	0.59
Diisopropyl	0.79
Di-n-propyl	1.19
Diisobutyl	1.84
Di-n-butyl	2.47
Di-2-ethyl-hexyl	19.16
Dioctyl	31.60

The above retention times are given only as a comparative illustration of the relative separation of the phthalates most frequently encountered under GLC conditions. For identification of GLC eluates by the mass spectrometer, the GLC separation of DEHP from other compounds that may be encountered in

extracts of plastics is shown in Figure 1; with rapid scanning of the eluted DEHP peak by the mass spectrometer, a mass spectrometer with an on-line computer that will correct for "background" bleeding from the GLC, the spectrum of DEHP may be rapidly obtained for confirmation of structure (Figure 2). Identification of the fragments with respect to the structures in Figure 2 is given in Figure 3.

For quantitation of GLC eluates that may not necessarily be easily separated by GLC, a mass spectrometer equipped with a multi-ion analyzer will also permit one to quantitate compounds at least at sub-nanogram levels. For instance, in the case of DEHP which gives a prevalent m/e fragment at 149 (cf., Figure 2), through the addition of deuterated DEHP as an internal standard in the sample, and containing four deuterium atoms in the aromatic ring, a second fragment characteristic of the protonated phthalic anhydride ion (m/e 149, Figures 2 and 3), will now appear as an m/e fragment of 153. Measurement of the heights of these two fragments at m/e 149 and 153 with the "multi-ion monitor," will yield a comparison of the concentrations of the two phthalate isomers (Figure 4) (cf., text for additional data).

Further details in regard to the characterization of other phthalate esters, particularly with respect to GLC separation, NMR, characteristic and mass spectrum-intensity peaks characteristic of the phthalates follows.

CHARACTERIZATION OF PHTHALATE ESTERS

In preparation for automated computerized identification of phthalate esters in sub-microgram quantities a series of phthalate were characterized spectroscopically, criteria of purity were established and optimal gas

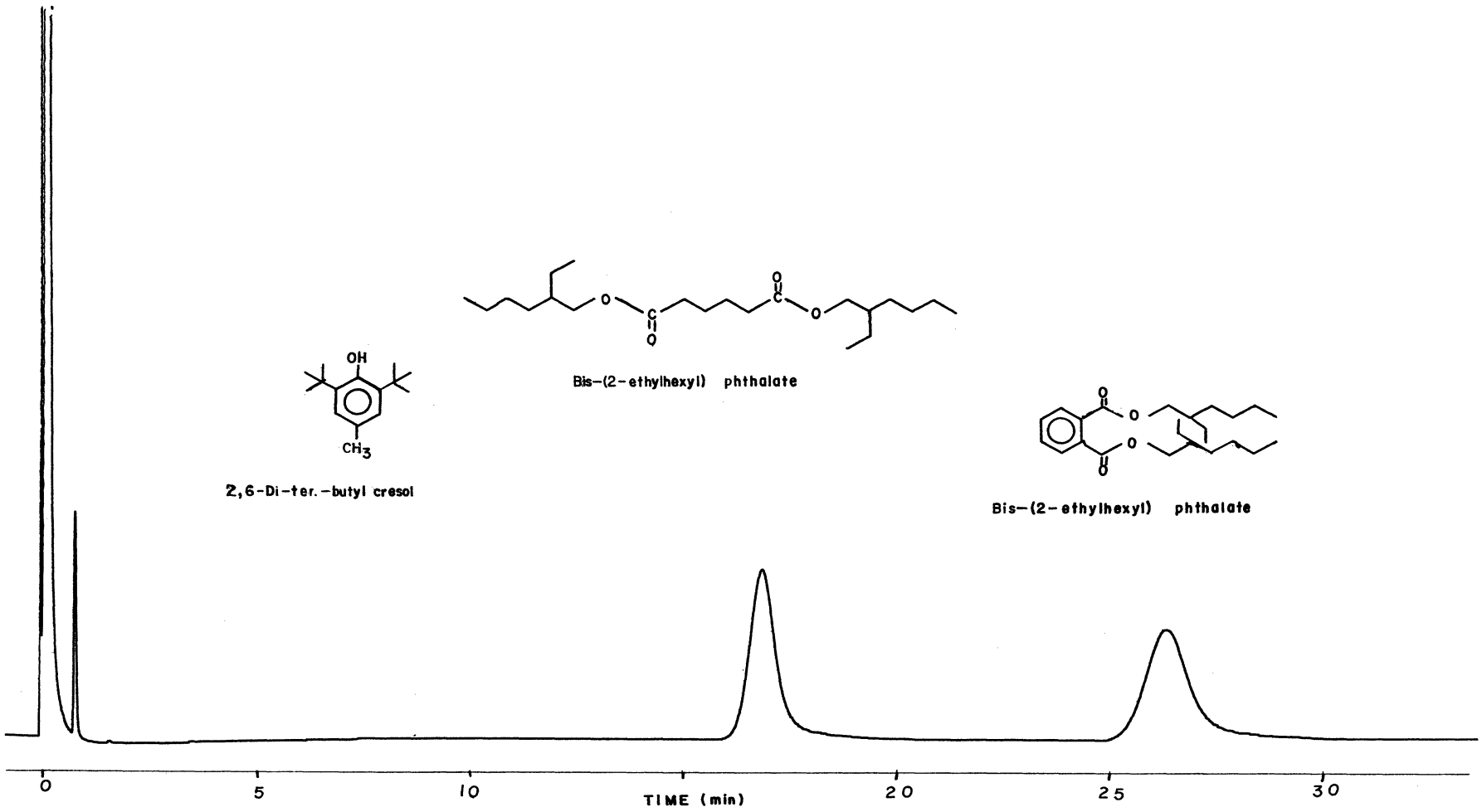


Figure 1. GLC separation of DEHP.

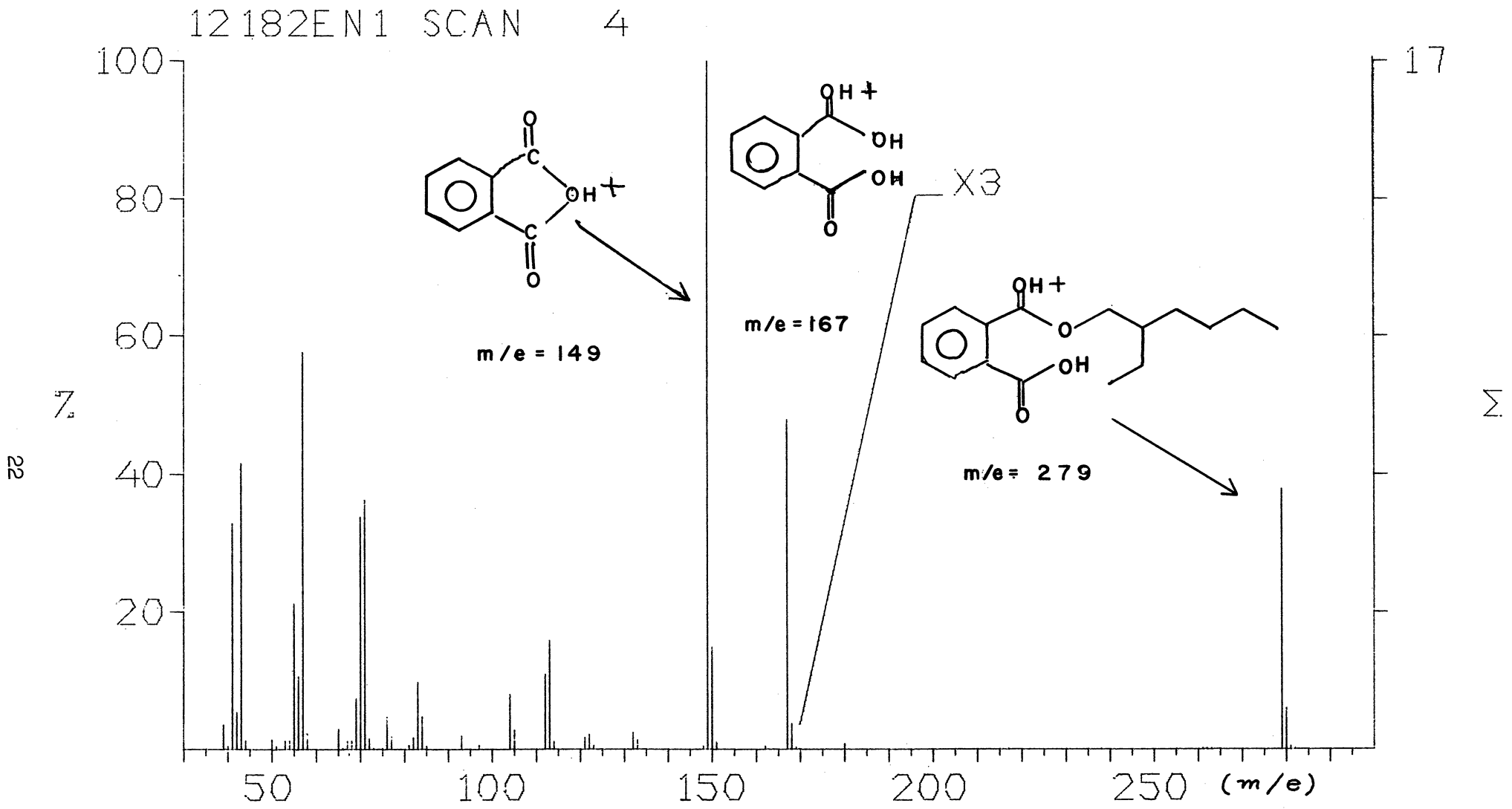


Figure 2. Mass spectrum of DEHP.

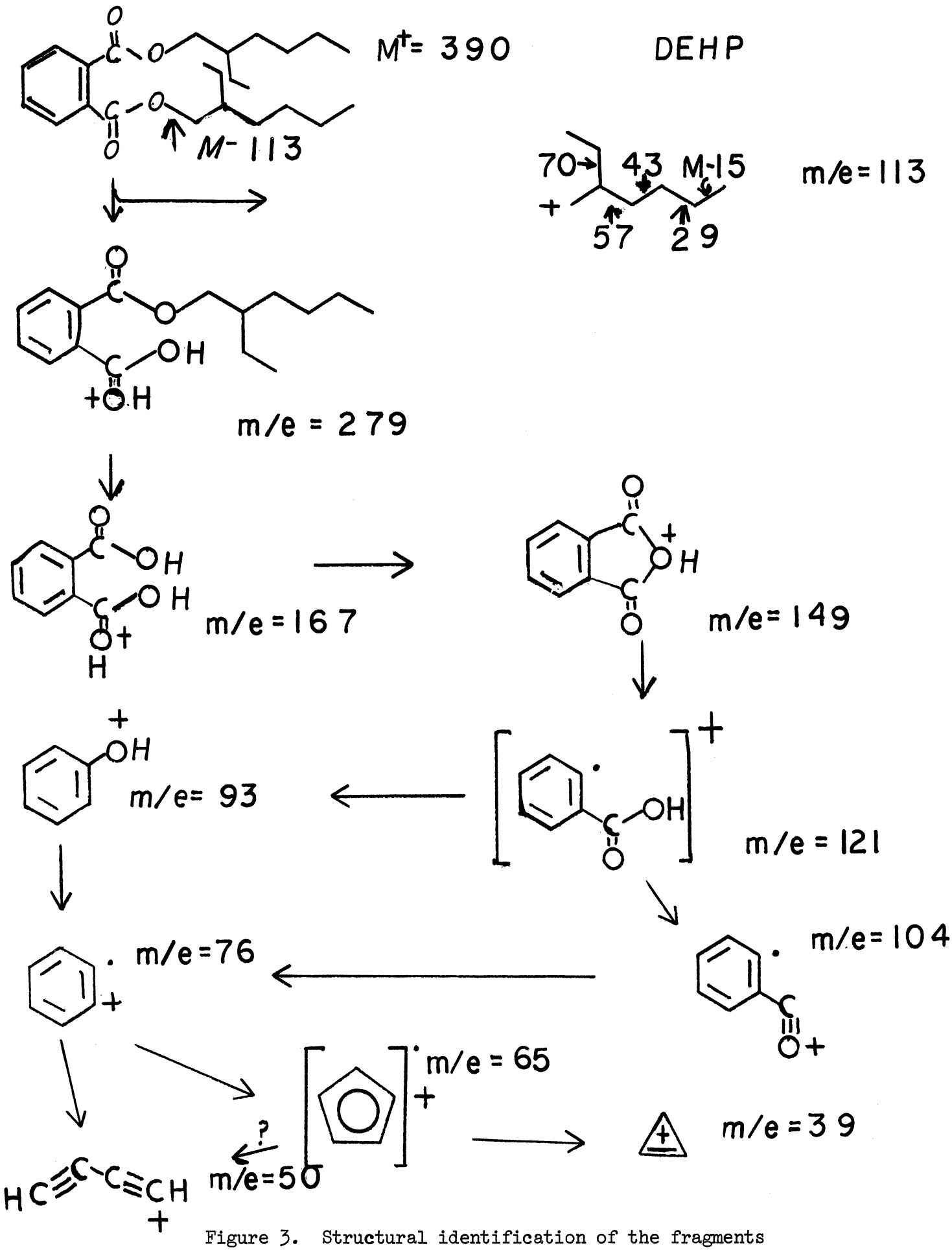


Figure 3. Structural identification of the fragments as they appear on the mass spectrum of Figure 2.

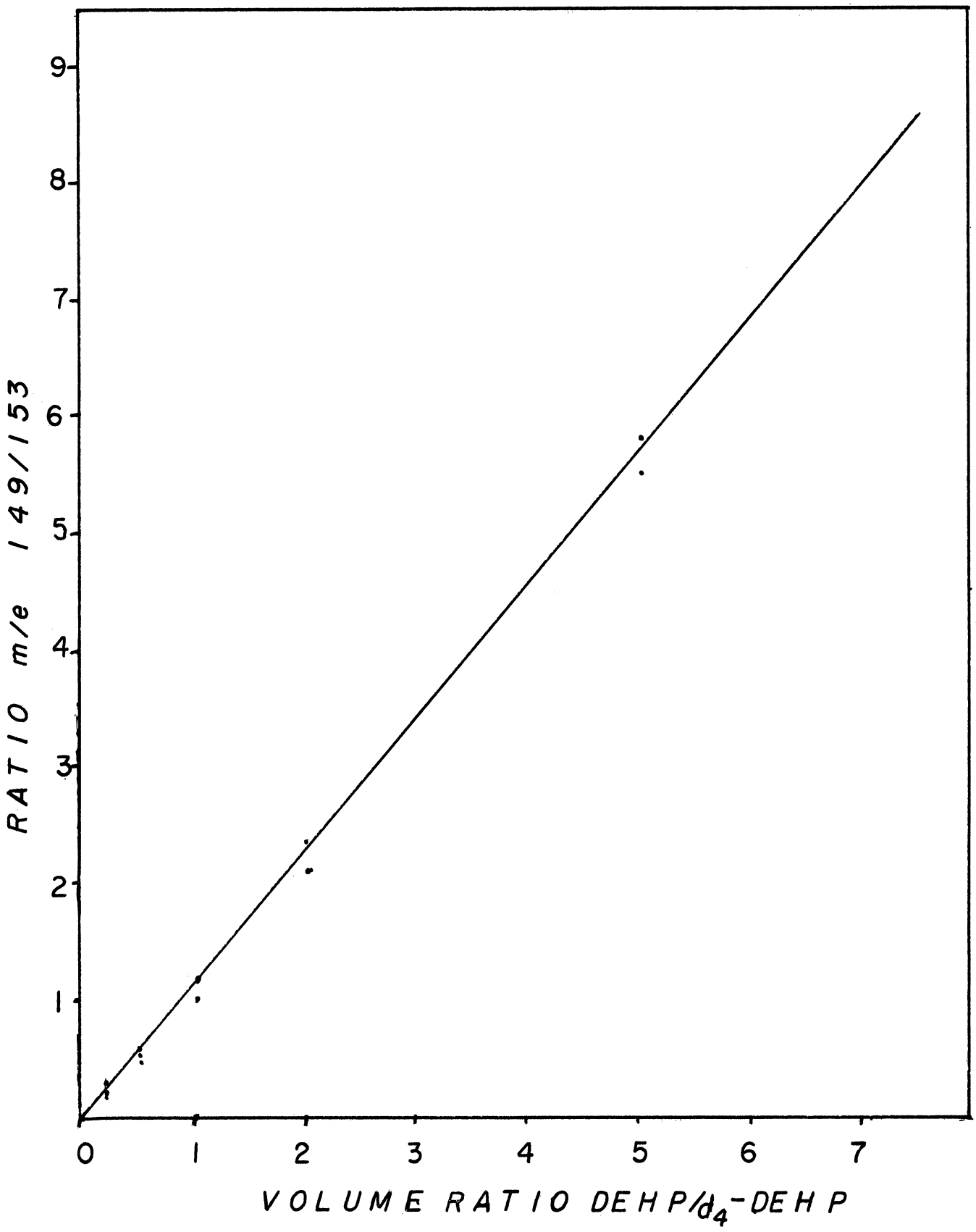


Figure 4. Multi-ion monitoring of DEHP with d_4-DEHP.

chromatographic parameters were determined. In several cases (e.g., dioctyl 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 G.C.-Mass Spectrometer data and were used in the cell culture studies reported below in the text.

IDENTIFICATION OF THE STRUCTURES OF COMMERICAL SAMPLES OF "DIOCTYL PHTHALATE"

Source	Structure
Matheson Colman and Bell	di-n-octyl phthalate
Eastman	di-n-octyl phthalate
Aldrich	bis(2-ethyl-hexyl) phthalate
Sargent-Welch	bis(2-ethyl-hexyl) 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)
7.6δ comp. mult. 1H
4.2δ 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)

7.6δ	comp. mult.	1H
4.3δ	trip. (J=6Hz)	1H
1.9-.7	complex	9H

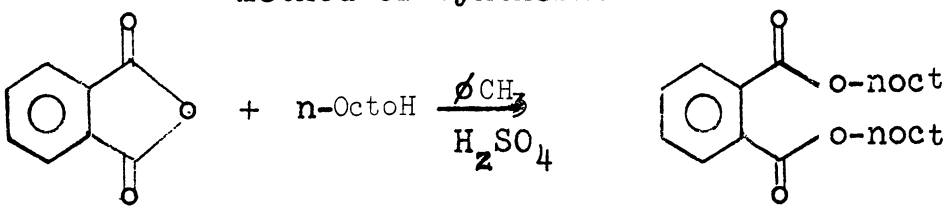
G.C. - mass spec.
3% SE-30
250° isothermal
Separator 220°
1 broad peak-ret. time 30.0 min.
(mass spec. for center of peak)

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

ADDENDUM II

MASS SPECTRAL IDENTIFICATION OF COMPOUNDS LEACHABLE FROM PLASTIC MEDICAL DEVICES

The following is an abstract of a paper that was presented at the Sixth Annual Central Regional Meeting of the American Chemical Society, April 21-24, in Detroit, Michigan.

> COMPOUNDS LEACHABLE FROM PLASTIC MEDICAL DEVICES. G. L. Ball, E. L. Johnson,* J. T. Groves,** and E. A. Napier,* Departments of Environmental and Industrial Health, *Biochemistry, and **Chemistry, The University of Michigan, Ann Arbor, Michigan 48104.

Blood stored in plastic bags has been shown to leach out plasticizers, such as di-2-ethyl-hexyl phthalate, which are found deposited in tissues subsequent to transfusion. There is also concern for kidney dialysis patients whose blood is regularly circulated through plastic devices. As part of a larger study of the accumulation, metabolism, and toxicity of common plasticizers, we analyzed five brands of commercially available tubing sets for use with disposable coil kidneys, two brands of artificial kidneys, and various tubing/kidney combinations for plasticizers, antioxidants, and other additives. To determine what compounds in each plastic might leach into plasma, pieces of tubing or kidney were digested with hexane and solutions were analyzed by gas chromatography/mass spectrometry (GC/MS). DEHP was found in all 5 tubing sets analyzed. Di-2-ethyl-hexyl adipate (DEHA) and butyl-carbo-butoxy-methyl phthalate were also identified in addition to an antioxidant 2,6-di-tert-butyl cresol (BHT). To determine if these compounds were actually leached out, 400-600 ml of human plasma was circulated through each set of tubing for 6 hours,

and samples were taken every hour. Analyses were performed by GC/MS after extractions. All additives found in the tubings were also found in the plasma extracts. During the 6-hour circulation, 10 mg DEHP, 4 mg DEHA, and 0.5 mg BHT entered the plasma.

ADDENDUM III

PLASMA EXTRACTION OF PLASTICIZERS FROM "MEDICAL GRADE" POLYVINYLCHLORIDE TUBING

Ronald E. Easterling, Elvin Johnson and E.A. Napier, Jr.[1]
(Introduced by John M. Weller)

Nephrology Division, Department of Internal Medicine,
University of Michigan Medical Center, Ann Arbor,
Michigan 48104

RUNNING TITLE: Plasticizers from PVC Tubing

Category: Biochemistry

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Mail proofs to:

Ronald E. Easterling
Nephrology Division
Department of Internal Medicine
The University of Michigan Medical Center
B2917 Interns Quarters
Ann Arbor, Michigan 48104

Up to 2 mg of di-2-ethylhexyl phthalate (DEHP) have been extracted in 5 hours from polyvinylchloride (PVC) tubing by a plasma perfusate (1). This highly lipid soluble compound has been shown to cause chemical peritonitis (1) and to be toxic in human tissue cultures (2). Little information is available regarding the extraction of DEHP and other phthalates from PVC tubings available commercially. In this study, we have demonstrated in vitro, that DEHP and di-2-ethylhexyl adipate (DEHA) are extracted in milligram quantities from tubings used for hemodialysis.

Methods and Materials. Tubings supplied to conduct blood to and from artificial kidneys were obtained from the following sources: Travenol Laboratories, Inc., Morton Grove, Illinois; Extracorporeal Medical Specialties Company, King of Prussia, Pennsylvania; Cobe Laboratories, Inc., Lakewood, Colorado; Life Med Corporation, Compton, California, and Sweden Artificial Kidney Supply Company, Seattle, Washington. In each case a specimen of tubing designed to deliver blood to the hemodialyzer was attached to the tubing designed for returning blood from the hemodialyzer. This system was then perfused with 500 to 700 ml of human plasma from a glass reservoir by a roller pump at 200 ml/min. The perfusate was maintained at 37^{o} by a water bath. Samples for phthalate determinations were taken from the reservoir.

Human plasma was obtained from blood collected in plastic bags in a blood bank. Blood specimens were immediately centrifuged and the plasma transferred to glass containers. The plasma was in contact with the plastic collection container for less than 30 minutes.

Plasma samples were extracted by the procedure of Folch et al. (3) with chloroform and methanol. The plasticizers were subsequently quantitated by computerized gas liquid chromatographic-mass spectrometer (4).

Results. DEHP was found in all samples of plasma before recirculation was begun (Table I). This phthalate probably originated from the plastic collection bags used to separate plasma from red blood cells. However, the presence of phthalates was not determined in the blood of these normal donors.

The DEHP level in the plasma perfusing the blood tubing progressively increased over the 5 to 6 hours of perfusion reaching levels as high as 35.9 μgm/ml (Table I). Table II lists the amount of DEHP extracted in the perfusate. The extraction rate for tubing sample A was markedly reduced during the second hour of recirculation when the pump was turned off. From 8.9 to 13.2 mg of DEHP were extracted from the 4 sets of tubing within six hours.

DEHA was found only in the perfusate for sample A. No DEHA was identified in the plasma before the perfusion began. Thereafter, hourly concentrations were 2.7, 3.7, 7.3, 8.5, and

9.7 μgm/ml. Like DEHP, the rate of extraction of DEHA into the plasma was markedly reduced during the hour without recirculation. The total amount of DEHA extracted after 5 hours was 4.2 mg.

Di-tert-butyl cresol (DTBC), an antioxidant, was also found in tubing D. None was identified in the plasma used for perfusion and only trace amounts were found after 1 and 2 hours of perfusion. Thereafter, the perfusate concentration of DTBC (μgm/ml) was 0.3 after 3 hours, 0.5 after 4 hours and 0.9 after 6 hours.

Discussion. Since extraction of DEHP was nearly linear in the tubing examined, even higher levels probably would be achieved with longer perfusion at 37°. The observation that DEHA was also found in one specimen and DTBC was also found in another spcimen, in addition to DEHP, suggests that at least 3 formulations of PVC tubings were involved. Accordingly, when the toxicology of such materials is in question, observations should be made for each source of the PVC tubing.

These results do not shed light on the biological significance of the phthalates extracted from the PVC tubings. However, the concentration of DEHP achieved in these experiments has been shown to be toxic for cells in tissue culture (2). There is also evidence suggesting toxicity of DEHP for an isolated liver preparation (1). These reports indicate the need for controlled studies with other conduit materials (such as

tetrafluorethylene polymer or silicone rubber) when PVC tubing is used to perfuse isolated organs.

The significance for the intact organism of phthalate extracted into protein containing solutions is less clear. While the rat metabolizes DEHP slowly (1,5) there is evidence suggesting that DEHP is rapidly metabolized in man (1,6). However, the potential toxicity of such metabolites is unknown.

Amounts of phthalate comparable to those recovered in the studies reported here must also be extracted from PVC tubings during hemodialysis for patients with renal failure. The loss of extracted phthalate through the cellulosic membrane of the hemodialyzer is probably minimal because of the limited water solubility of these compounds (7). Although the total amounts of phthalate which are extracted in vitro and which could be given intravenously during hemodialysis are considerably below oral toxic doses (8), the ratio of urine to fecal 14-C is higher after intravenous than after oral administration of 14C-labeled DEHP (5), suggesting that in anephric patients more DEHP and its metabolites accumulate after intravenous infusion than after oral ingestion. Furthermore, hemodialysis patients may be exposed to the PVC tubing of the artificial kidney three times a week for many years. Since patients undergoing chronic dialysis do not have a normal life span (9) and chronic phthalate toxicity cannot be excluded in man, further study to clarify these points in vivo is warranted.

Summary. Up to 12 mg of DEHP and 8 mg of DEHA were extracted from "medical grade" polyvinylchloride tubing by human plasma recirculated for 6 hours at 37°. The demonstrated toxicity of DEHP for *in vitro* systems suggests that this type of tubing should be evaluated for toxic effects on *in vitro* organ perfusion systems. The significance of these observations for medical applications has not been determined.

TABLE I. DEHP CONCENTRATION IN PLASMA PERFUSING PVC TUBING[a]

TUBING	HOURS OF PERFUSION						
	0	1	2	3	4	5	6
A	3.0	5.6	5.7[b]	10.7	13.6	15.5	-
B	1.1	6.2	10.8	16.5	18.5	28.9	27.5
C	2.0	5.6	8.7	12.9	16.7	24.0	28.7
D	1.4	3.9	6.3	9.2	12.8	-	21.0
E	0.5	5.4	10.4	18.4	-	27.0	35.9

[a] μgm/ml

[b] Recirculation stopped during second hour.

TABLE II. DEHP EXTRACTED BY PLASMA PERFUSING PVC TUBING[a]

TUBING SOURCE	HOURS OF PERFUSION					
	1	2	3	4	5	6
A	1.15	1.16	3.1	4.15	4.45	-
B	2.0	3.8	5.9	6.6	9.9	9.6
C	1.6	2.65	4.15	5.35	7.6	8.9
D	1.4	2.7	4.3	6.2	-	10.15
E	2.15	4.25	7.45	-	10.2	13.2

[a]Cumulative amounts of DEHP (mg) extracted by 500 to 700 ml of human plasma.

References:

1. Jaeger, R.J. and Rubin, R.J. Environmental Health Perspectives. DHEW Publication No. (NIH) 72-318, Research Triangle Park, North Carolina. January 1973, pp. 95-102.

2. Jones, A., Kahn, R. and Napier, E. Nature (London), In Press.

3. Folch, J., Rees, M. and Sloane, G.H. J. Biol. Chem. 226, 497 (1957).

4. Sweely, C.C., Ray, B.D., Wood, W.I., Holland, J.F. and Krichivsky, M.I. Anal. Chem. 42, 1505 (1970).

5. Albro, P.W., Thomas, R. and Fiskbein, L. J. Chromatogr. 76, 321 (1973).

6. Jaeger, R.J. and Rubin, R.J. N. Engl. J. Med. 287, 1114, (1972).

7. Schreiner, G.E. Trans. Amer. Soc. Artif. Intern. Organs, 16, 544 (1970).

8. Burton, B.T., Krueger, K.K. and Bryan, F.A., Jr. J. Amer. Med. Ass. 218, 718 (1971).

9. Krauskopf, L.G. Environmental Health Perspectives. DHEW Publication No. (NIH) 72-318, Research Triangle Park, North Carolina. January 1973, pp. 61-72.

Phthalate Ester Toxicity in Human Cell Cultures

Adelaide E. Jones

Raymond H. Kahn

Department of Anatomy

The University of Michigan

John T. Groves

Department of Chemistry

The University of Michigan

Edward A. Napier, Jr.

Departments of Biological Chemistry and Internal Medicine

The University of Michigan

Correspondence:

Raymond H. Kahn

5793 Medical Science II

Department of Anatomy

The University of Michigan

Ann Arbor, Michigan 48104

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ABSTRACT

Di-2-ethylhexyl phthalate and butyl glycolyl butyl phthalate, plasticizers which can be leached into blood from polyvinyl chloride containing medical devices, cause significant growth inhibition in cultures of the human diploid cell strain WI-38. The ID_{50} values for di-2-ethylhexyl phthalate and butyl glycolyl butyl phthalate are 70μM and 12μM respectively for WI-38 cells. Toxic effects are greater in a replicating cell population than in a nonreplicating, confluent cell layer. WI-38 cells which have been grown in 160μM di-2-ethylhexyl phthalate for 3 days, and subsequently subcultured into control medium, show only 60% of control growth after 5 days in control medium. Cells treated with 14μM butyl glycolyl butyl phthalate for 3 or 5 days exhibit growth equivalent to the controls when subcultured into control medium. Toxic levels for di-2-ethylhexyl phthalate are within the range of levels found in blood which has been stored in polyvinyl chloride blood bags for up to 21 days at 4° C. ID_{50} values are reported for several other phthalate esters and for two nonphthalide compounds which are leachable from certain polyvinyl chloride plastic medical devices.

INTRODUCTION

Phthalates, which are used as plasticizers for many polyvinyl chloride (PVC) items in medical and laboratory use, have recently come under closer investigation (Autian, 1973). It has been shown that measurable levels of di-2-ethylhexyl phthalate (DEHP)* accumulate in blood stored in PVC bags for up to 21 days at 4° C (Jaeger and Rubin, 1970a), and in anticoagulant solution stored in PVC bags (Jaeger and Rubin, 1970a, Guess et al., 1967). Also, DEHP has been found in the tissue of deceased patients who had recently received transfusions (Jaeger and Rubin, 1972). Certain formulaions of PVC tubing used in hemodialysis units have been shown to release DEHP into circulating plasma (Jaeger and Rubin, 1970b). Other tubing formulations have revealed the presence of another plasticizer, butyl glycolyl butyl phthalate (BGBP) (Jaeger and Rubin, 1970b, Trimble et al., 1966).

Both DEHP and BGBP have low acute toxicity in rats (Singh et al., 1972, Calley et al., 1966) and show some teratogenic effects when administered to rats in large doses (Singh et al., 1972). However, it is important to study the subacute toxicity of these compounds by tissue culture assay. We have chosen to use the human diploid cell strain WI-38 (Hayflick, 1965) to study changes in total cell protein and in gross morphology. Both DEHP and BGBP, which we have rigorously purified, are added directly to the culture medium similar to unpublished procedures on mouse fibroblasts (Autian, 1973). While previous in vitro studies have described an agar overlay method (Guess et al.,1965, Guess and Haberman, 1968), the validity of this approach with DEHP and BGBP is open to question. These compounds have a very low water solubility, and may not completely

* The more correct nomenclature is bis (2-ethylhexyl) phthalate.

permeate the agar layer. One study using this method noted that the most soluble phthalates were the most toxic (Calley et al., 1966), while no toxicity was reported for DEHP. Our experiments provide a more direct approach for determining the effects of phthalate esters on human cells.

METHODS

Human diploid cell strain WI-38[1] was grown in Basal Medium Eagle[2] with 10% calf serum[3]. The cultures used for protein determination (Fig. 2) were grown in Falcon T-25 flasks[4] which were incubated at 37° C and maintained at pH 7.2-7.4. Cell protein was determined by the method of Oyama and Eagle (1956). Cells grown on cover slips were stained with oil red O and hematoxylin (Fig. 1).

We have found that both DEHP[5] and BGBP[6] solublize more readily in calf serum than in the culture medium alone. Thus, both phthalates were first stirred into the calf serum moiety of the medium for 22 hours, which was, in turn, stirred into the culture medium for an additional 22 hours. Even by this method, the compounds were incompletely solublized, and were visible as an emulsion. To determine the true amount in solution, the emulsion was removed by gentle centrifugation, and the aqueous samples were extracted by the Folch technique (Folch et al., 1957). Quantitation of these samples was performed by Gas Liquid Chromatography (GLC) using a 1% SE30 column (1.8m by 4mm) on 80-100 mesh Chromosorb-W, at 200° C. Assays were also conducted on a computerized GLC mass spectrophotometer (Sweeley et al., 1970)

The principle phthalates used in this study were DEHP and

BGBP. However, the following other phthalates (Table 1) were studied to determine ID_{50} values (dose which cause 50% growth inhibition in tissue culture): di-n-butyl phthalate[7], di-iso-butyl phthalate[8], dimethoxyethyl phthalate[9], and di-n-octyl phthalate[10]. Two nonphthalide, PVC leachable compounds tested were di-2-ethylhexyl adipate[11] and 2,6-di-ter-butyl cresol[12]. All compounds were chromatographed and rigorously purified using Woelm silica gel[13] with a benzene: ethyl acetate ratio of 95:5.

RESULTS

GLC analysis reveals that concentrations of 0.25 mM, 0.50 mM, and 1.00 mM DEHP added to the culture medium result in soluble concentrations of approximately 51 μM, 69 μM, and 160 μM respectively. The presence of an emulsion at all three concentrations and the differences in solubility indicate that the system has not reached final equilibrium. Our measurements of soluble concentrations before addition to the cell layer, and after 5 days in culture, demonstrate that there is some further solublization of DEHP during culture, both within the ranges of the figures reported here.

Cell treated with 160 μM DEHP for 6 days (Fig. 1b) show a decreased cell density when compared to controls (Fig. 1a). The flasks treated with 51 μM and 69 μM DEHP show decreased cell protein (Fig. 2b) and a longer generation time, although these cells are still viable at the end of 9 days of treatment. The differences in protein observed between concentrations are statistically significant. Cells treated with 160 μM DEHP are no longer viable by day 9. From these data, it is possible to determine an ID_{50} value of 70 μM for WI-38 cells. Our ID_{50} value is calculated

by plotting the percent of control protein after nine days of phthalate treatment vs. each phthalate concentration. The phthalate concentration at 50% of control protein is recorded from this plot.

Concentrations of 0.25 mM, 0.50 mM, and 1.00 mM BGBP added to the culture medium result in soluble concentrations of 7 μM, 10 μM, and 14 μM BGBP respectively. The appearance of cells treated with 14 μM BGBP for 6 days (Fig. 1b) is very similar to that shown by cells treated with 160 μM DEHP for 6 days. The ID_{50} value for BGBP in WI-38 cells is 12 μM.

To test the effects of DEHP and BGBP on high density cell populations, the cells are allowed to reach an imbricated confluency before the addition of phthalate. At these densities, very little cell division occurs. While these confluent populations are affected by DEHP and BGBP, the effects on cell protein are proportionately less than those which are exhibited by replicating populations (Fig. 2). Structurally, the cells appear thinner after several days of treatment, and open spaces appear in the previously confluent layer. After 9 days of treatment with 69 μM DEHP, confluent populations exhibit 61% of control protein, whereas replicating populations show only 50% of the control value. Treatment for 9 days with 14 μM BGBP results in 72% of control protein in confluent populations, in contrast to 48% of control protein in replicating populations.

To determine the reversibility of DEHP and BGBP effects on cultured cells, cells grown in the presence of either compound for 3 and 5 days were subsequently subcultured at equal viable cell densities in the control medium. After 5 days in the control medium, the flasks were washed and protein determined. Cells grown for 3 days in 160 μM DEHP showed only 60% of the control growth after

subculture in the control medium. Moreover, the cells treated with DEHP did not fully extend their cytoplasmic processes until 2 days later, the normal time being less than 6 hours. These data suggest possible membrane damage. In contrast, cells treated with BGBP at 14 μM concentrations for 3 and 5 days did not show decreased viability or a change in membrane attachment after subculture.

Table 1 shows that the ID_{50} values for other phthalate esters are also in the micromolar range. Two nonphthalide leachable compounds which we have recently detected in the plasma perfusate from a hemodialysis apparatus, 2,6-di-tert-butyl cresol, and di-2-ethylhexyl adipate (Jaeger and Rubin, 1973a), are also toxic at low levels. However, the water solubility of all but di-n-butyl phthalate and dimethoxyethyl phthalate is very low, thus, keeping the danger of most of these compounds within the ranges of subtle toxicity. Di-n-butyl phthalate is the most toxic of this series.

DISCUSSION

Our calculated ID_{50} value of 70 μM for DEHP in WI-38 cells is very similar to the value of 50 μM reported by Autian's laboratory (Autian, 1973) for mouse L-929 cells. In addition, our observation that nonreplicating WI-38 populations are less affected than replicating ones is in support of Dillingham and Autian (1973). They found that nonreplicating L-929 cells are more protein stable than replicating ones when treated with dimethoxyethyl phthalate. This may be linked to the findings by Warren and Glick (1968) that there is a protein turnover rate in both dividing and nondividing cells, which is independent of mitosis. Dividing cells have an additional protein turnover during mitosis, which creates membrane

instability and a higher susceptibility to toxicants at this stage.

Some of the major concerns which have arisen over the use of DEHP, in particular, are the findings that DEHP does not appear to be easily metabolized. Detectable amounts have been found in human tissue (Jaeger and Rubin, 1972), fish tissue (Mayer et al., 1971), and in bovine heart tissue (Nazir et al., 1971). Studies of the absorption and metabolism of related compounds, 2-alkylalkanoates, in rats (Saladin and Napier, 1967), suggest that steric hindrance of the 2-ethyl group in DEHP could alter the absorption and hydrolysis rates of this compound. Thus, it is not unreasonable to expect that some DEHP could be deposited in the tissues.

In blood, the concentration of DEHP has been reported to reach concentrations of approximately 134 μM following storage for 21 days at 4° C in PVC bags (Jaeger and Rubin, 1972). Equally important, 486 μM DEHP has been detected in platelet concentrates stored at 22° C for only 2 days (Jaeger and Rubin, 1973b). These concentrations are comparable to those found in this study which inhibit WI-38 cell growth completely, causing cell degeneration and death in tissue culture. Our studies of BGBP and of other phthalate esters, as well as two nonphthalide compounds found leachable from medical devices, indicate a similar toxic effect. Although there does not appear to be an immediate clinical problem, there is evidence of subtle toxicity from many phthalate esters found in PVC containing plastics, and the possibility of tissue accumulation of DEHP, in particular.

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Fig. 1. Monolayer culture of WI-38 cells exposed to 160 μM DEHP for 6 days (b) as compared to the effect of the control medium (a). Oil red O, hematoxylin stain.

Fig. 2. Protein content of cells treated with BGBP (a) and DEHP (b). Each point represents the mean value for 3 sample flasks. Brackets indicate ± 1.5 standard error of the mean.

TABLE 1. ID_{50} VALUES FOR A SERIES OF PHTHALATE ESTERS AND LEACHABLE NONPHALIDE COMPOUNDS USING WI-38 CELLS

Compound	Molecular Weight	ID_{50}[a]	Solubility Moles/Liter
PHTHALATE ESTERS			
Di-n-butyl phthalate	278	135 μM	0.008
Di-iso-butyl phthalate	278	85	very low
Dimethoxyethyl phthalate	282	3500	0.040
Butyl glycolyl butyl phthalate	336	12	very low
Di-n-octyl phthalate	391	170	very low
Di-2-ethylhexyl phthalate	391	70	very low
NONPHTHALIDE COMPOUNDS			
Di-2-ethylhexyl adipate	371	32	very low
2,6-Di-tert-butyl cresol	220	67	very low

a. media concentration which cause 50% growth inhibition in tissue culture.

FOOTNOTES

1. Obtained from L. Hayflick, Stanford, California.
2. Gibco, formula G-13, Grand Island, New York.
3. Flow Laboratories, Rockville, Maryland.
4. Falcon Plastics, Oxnard, California.
5. Aldrich Chemical Co., Milwaukee, Wisconsin.
6. Matheson, Coleman, and Bell, Norwood, Ohio.
7. Sargent-Welch, Skokie, Illinois.
8. Aldrich Cehmical Co., Milwaukee, Wisonsin.
9. Fluka AG, Chemische Fabrik, Germany.
10. Eastman Kodak Co., Rochester, N.Y.
11. Synthesized from 2-ethylhexanol (Aldrich) and adipic acid (Eastman Kodak) and purified as above.
12. Eastman Kodak Co., Rochester, N.Y.
13. M. Woelm, Eschwege, Germany, U.S. distributor ICN Pharmaceuticals, Cleveland, Ohio.

REFERENCES

Autian, J. (1973). Toxicity and Health Threats of Phthalate Esters: Review of the Literature. Environ. Health Perspectives Exper. Issue 4,3-25.

Calley, D.J., Autian, J., and Guess, W.L. (1966). Toxicology of a Series of Phthalate Esters. J. Pharm. Sci. 55,158-162.

Dillingham, E.O., and Autian, J. (1973). Teratogenicity, Mutagenicity, and Cellular Toxicity of Phthalate Esters. Environ. Health Perspectives Exper. Issue 3,81-89.

Folch, J., Lees, M., and Stanley Sloane, G.H. (1957). A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues. J. Biol. Chem. 226,497-509.

Guess, W.L., Rosenbluth, S.A., Schmidt, B., and Autian, J. (1965). Agar Diffusion Method for Toxicity Screening of Plastics on Cultured Cell Monolayers. J. Pharm. Sci. 54,1545-1547.

Guess, W.L., Jacob, J., and Autian, J. (1967). A Study of Polyvinyl Chloride Blood Bag Assemblies. I. Alteration or Contamination of ACD Solutions. Drug Intel. 1,120-127.

Guess, W.L., and Haberman, S. (1968). Toxicity Profiles of Vinyl and Polyolefinic Plastics and Their Additives. J. Biomed. Mater. Res. 2,313-335.

Hayflick, L. (1965). The Limited In Vitro Lifetime of Human Diploid Cell Strains. Exp. Cell Res. 37,614-636.

Jaeger, R.J., and Rubin, R.J. (1970a). Contamination of Blood Stored in Plastic Packs. Lancet 2,151.

Jaeger, R.J., and Rubin, R.J. (1970b). Plasticizers from Plastic Devices: Extraction, Metabolism, and Accumulation by Biological Systems. Science 170,460-462.

Jaeger, R.J., and Rubin, R.J. (1972). Migration of a Phthalate Ester Plasticizer from Polyvinyl Chloride Blood Bags into Stored Human Blood and its Localization in Human Tissues. N. Eng. J. Med. 287,1114-1118.

Jaeger, R.J., and Rubin, R.J. (1973a). Extraction, Localization, and Metabolism of Di-2-ethylhexyl Phthalate from PVC Plastic Medical Devices. Environ. Health Perspectives Exper. Issue 3,95-102.

Jaeger, R.J., and Rubin, R.J. (1973b). Di-2-ethylhexyl Phthalate, a Plasticizer Contaminant of Platelet Concentrates. Transfusion 13,107-108.

Mayer, F.L., Stalling, D.L., and Johnson, J.L. (1971). Phthalate Esters: An Environmental Contamimant. Report, Fish-Pesticide Research Laboratory, Bureau of Sport Fisheries and Wildlife, United States Department of the Interior, Columbia, Missouri.

Nazir, D.J., Alcaraz, A.P., Bierl, B.A., Beroza, M., and Nair, P.P. (1971). Isolation, Identification, and Specific Localization of Di-2-ethylhexyl Phthalate in Bovine Heart Muscle Mitochondria. Biochem. 10,4228-4232.

Oyama, V.I., and Eagle, H. (1956). Measurement of Cell Growth in Tissue Culture with a Phenol Reagent (Folin-Ciocalteau). Proc. Soc. Exp. Biol. Med. 91,305-307.

Saladin, T.A., and Napier, E.A., Jr. (1967). Properties and Metabolism of 2-alkylakanoates. III: Absorption of Methyl and Ethyl 2-Methylpalmitate. J. Lipid Res. 8,342-349.

Singh, A.R., Lawrence, W.H., and Autian, J. (1972). Teratogenicity of Phthalate Esters in Rats. J. Pharm. Sci. 61,51-55.

Sweeley, C.C., Ray. B.D., Wood, W.I., Holland, J.F., and Kritchevsky, M.I. (1970). On-line Digital Computer System for High Speed Single Focusing Mass Spectrometry. Anal. Chem. 42,1505-1516.

Trimble, A.D., Goldman, B.S., Yao, J.K., Kovats, L.K., and Bigelow, W.G. (1966). Plastics - A Source of Chemical Contamination in Surgical Research. Surgery 59,857-859.

Warren, L., and Glick, M.C. (1968). Membranes of Animal Cells. II. The Metobolism and Turnover of the Surface Membrane. J. Cell Biol. 37,729-746.

INDEX TERMS

Phthalate esters = phthalic acid esters

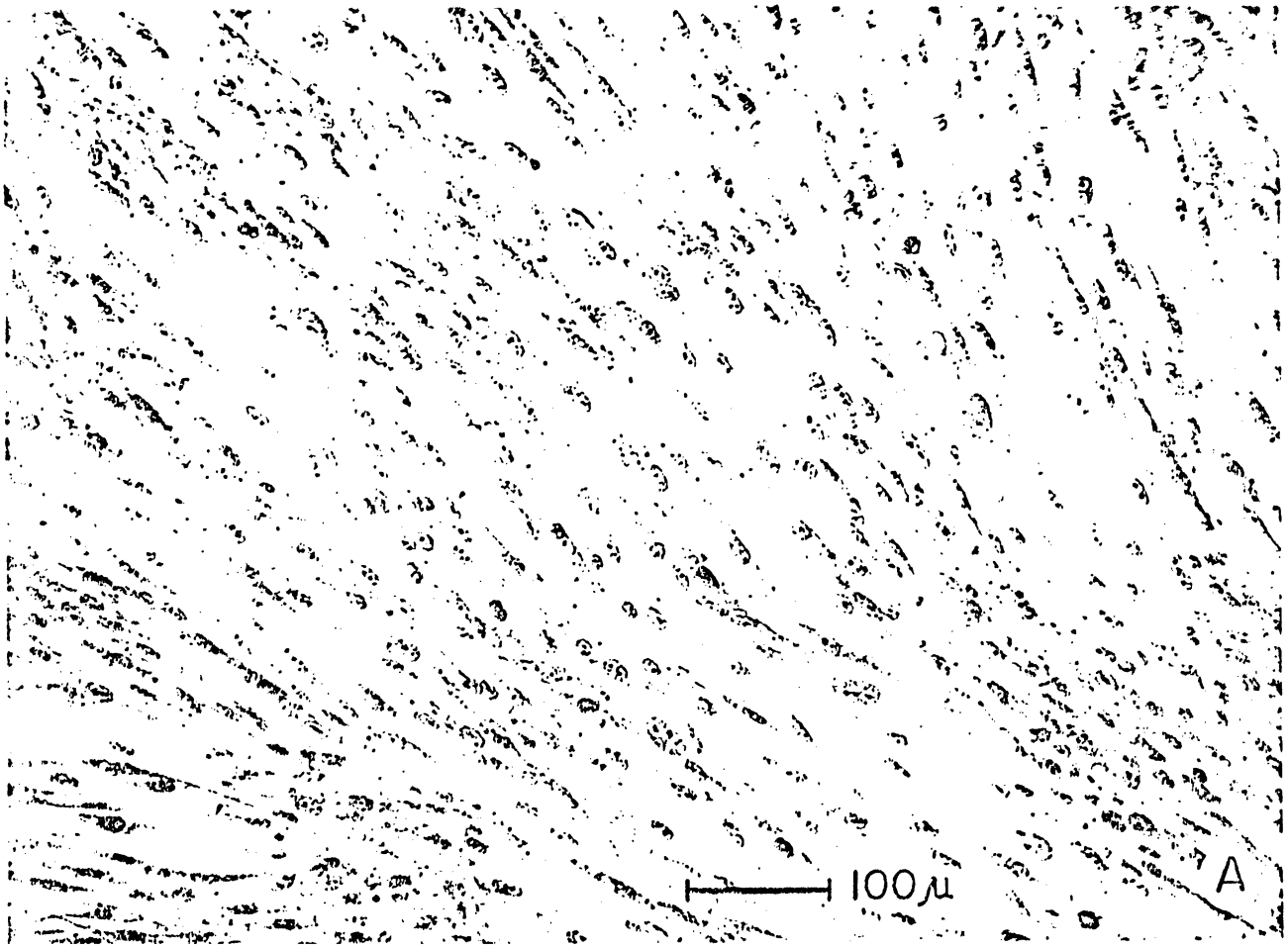
Di-2-ethylhexyl phthalate = bis-2-ethylhexyl phthalate

Butyl glycolyl butyl phthalate = butyl carbobutoxymethyl phthalate

Plasticizers

WI-38 human diploid cell strain

Polyvinyl chloride plastic



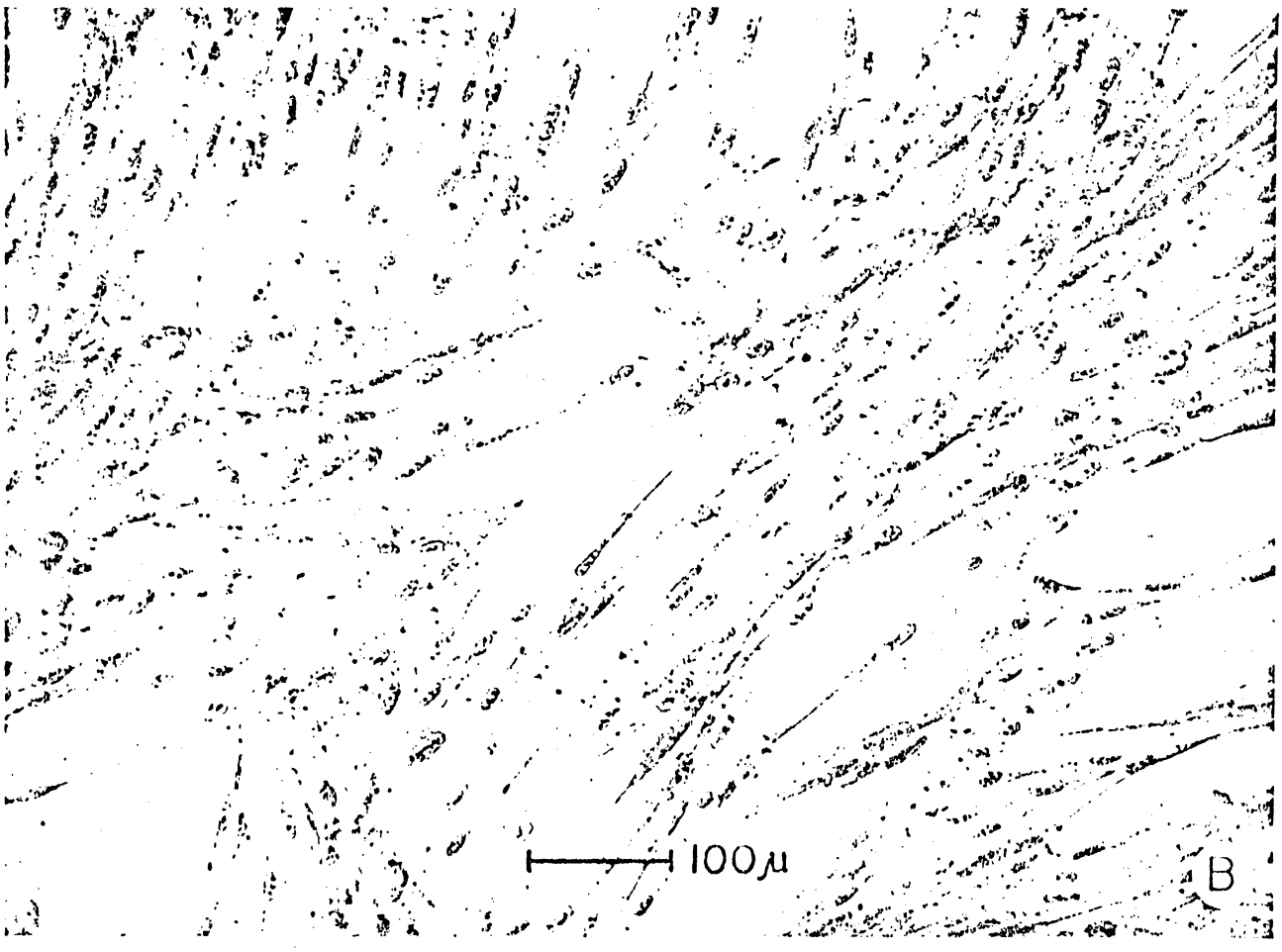
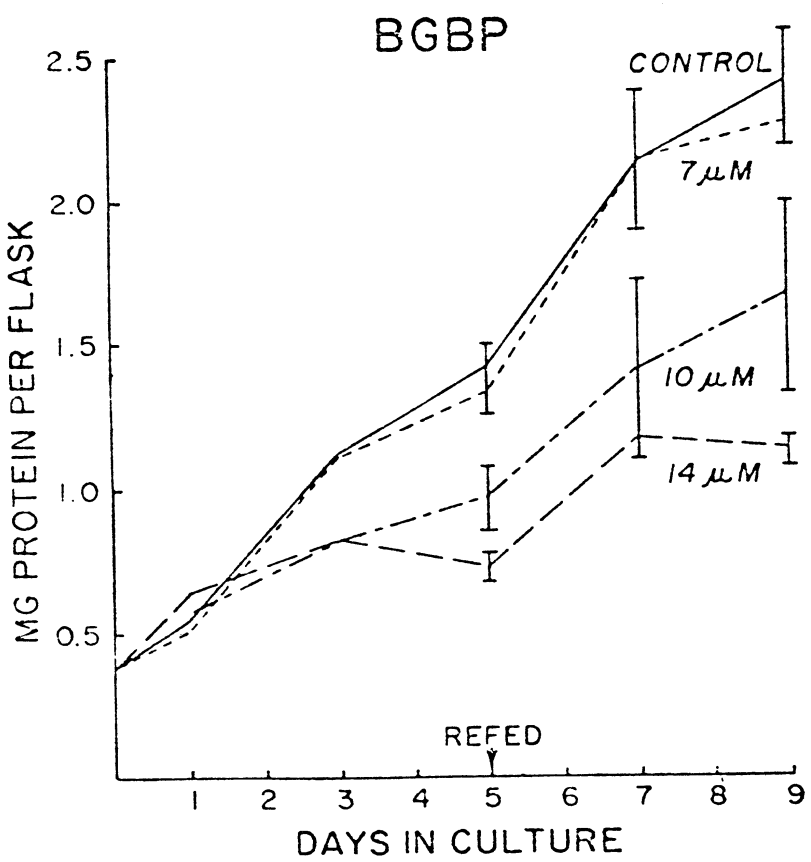
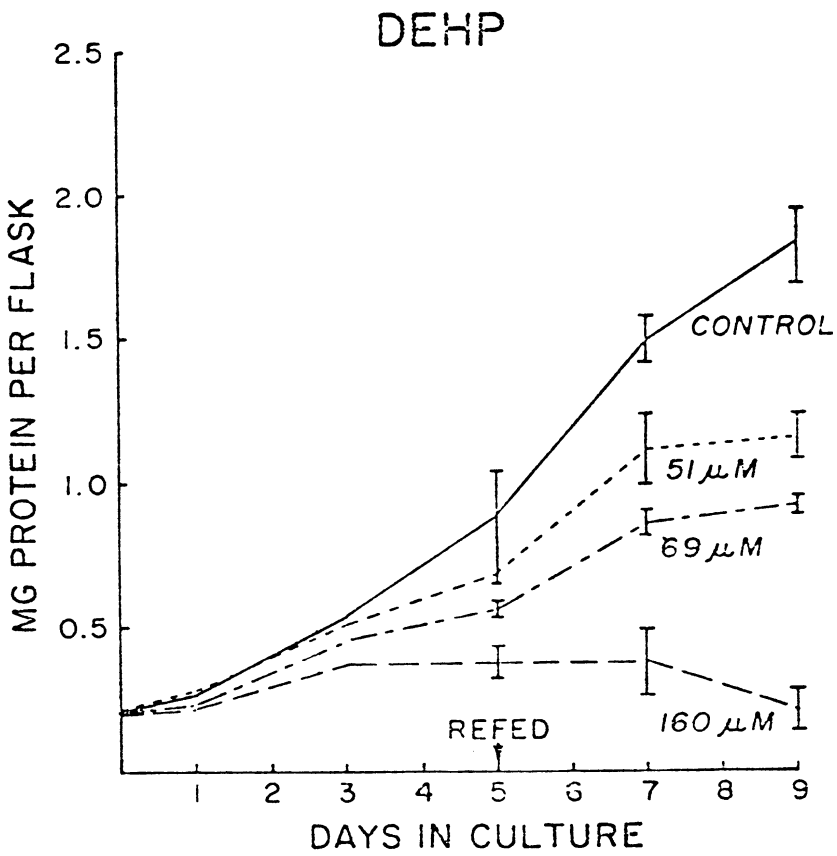


Figure 1


BGBP
CONTROL
7 μM
10 μM
14 μM
REFED
MG PROTEIN PER FLASK
DAYS IN CULTURE
2.5
2.0
1.5
1.0
0.5
1 2 3 4 5 6 7 8 9

A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CONTROL
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69 μM
160 μM
REFED
MG PROTEIN PER FLASK
DAYS IN CULTURE
2.5
2.0
1.5
1.0
0.5
1 2 3 4 5 6 7 8 9

B

FIGURE 2

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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 in vivo and in vitro metabolic studies in humans and human cell cultures, double-labeled phthalate esters have been synthesized with both non-radioactive ($^{13}C;^{2}H$) and radioactive ($^{14}C;^{3}C$) isotopes for use, respectively, in these studies now in progress. In vitro studies of the toxicity of phthalates (nominally 0.1 to 1.0 mM conc.) on human, fetal fibroblast cultures (WI-38) have demonstrated clear dose-response related curves over a period of up to 9 days' incubation, with respect to decreased protein synthesis. Perfusion of human plasma in vitro through hemodialysis units ordinarily used with renal patients, has shown an incremental release fo di-2-ethyl-hexyl phthalate (DEHP), attaining a concentration of up to 3.6 mg% over a 6-hour period, the normal time period that patients are exposed to these units. Of 116 analyses completed to date from human tissues obtained at necropsy (heart, liver, spleen, kidney, lung, brain, omentum), measurable levels of DEHP have been shown to occur (27%). Further data are needed, however, to correlate the significance of these data with medical histories (e.g., transfusions, hemodialysis, sex, age, etc.).

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; occurrence in human tissues.

17b. Identifiers/Open-Ended Terms

17c. COSATI Field/Group

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