T H E U N I V E R S I T Y O F M I C H I G A N

MEDICAL SCHOOL
Department of Biological Chemistry

Progress Report

ACCUMULATION, TOXICITY, AND METABOLISM OF COMMON PLASTICIZERS IN HUMANS

October 1973 - January 1974

Edward A. Napier, Jr.

DRDA Project 011711

under contract with:

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
U.S. PUBLIC HEALTH SERVICE
NATIONAL HEART AND LUNG INSTITUTE
CONTRACT NO. NHLI-73-2936-B
BETHESDA, MARYLAND

administered through:

DIVISION OF RESEARCH DEVELOPMENT AND ADMINISTRATION ANN ARBOR

January 1974

TABLE OF CONTENTS

	Page
PRINCIPAL INVESTIGATORS	iii
SUMMARY	iv
SYNTHESES OF LABELLED COMPOUNDS	1
EFFECTS OF BCBP ON BLOOD COAGULATION IN VITRO	4
MONITORING OF HUMAN TISSUES FOR THE PRESENCE OF PLASTICIZERS	6
MASS SPECTRAL IDENTIFICATION OF COMPOUNDS LEACHABLE FROM PLASTIC MEDICAL DEVICES	10
PLASMA EXTRACTION OF PLASTICIZERS FROM "MEDICAL GRADE" POLYVINYLCHLORIDE TUBING	11
PHTHALATE ESTER TOXICITY IN HUMAN CELL CULTURES	19

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-ethylhexyl phthalate (DEHP) double-labeled with both stable isotopes (carbon-13 and deuterium) and radioactive isotopes (carbon-13 and tritium), has been completed, and distributed to other contractors at their request.

In the coagulation studies with human platelets, results similar to those previously reported for DEHP, have been found with butylcarbobutoxy phthalate (BCBP). As discussed at the last Contractors' meeting, however, these studies will have to be repeated under a different experimental design, and are now in progress.

In our monitoring program for the occurrence of DEHP in human tissues obtained at necropsy, it is interesting to note that DEHP was detected in nine of the ten livers, seven out of ten spleens, four out of eight hearts, three out of eight lungs, and five out of eight kidneys analyzed to date. It sould be emphasized these are very preliminary data and no conclusions are appropriate at this time. Similarly, the limited quantitative data does not permit us to correlate these data with medical histories.

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 Sjouris 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.

The compounds found leachable from plastic medical devices that we identified by mass spectrometry will be presented before the mid-western spring meeting of the American Chemical Society. The abstract appears on page 10.

The quantitation of the leakage of plasticizers into human plasma circulated through various commercial hemodialysis units has been completed for five units; and pages 11 through 18 essentially represent a copy of the manuscript submitted for publication in Proceedings of the Society for Experimental Biology and Medicine.

Finally, data on the toxicity of the plasticizers on human cell cultures have been summaried into a manuscript that has been submitted for publication in Nature; pages 19 through 31 is a copy of this manuscript.

SYNTHESES OF LABELLED COMPOUNDS

1. Bis(2-ethylhexyl)phthalate-$\underline{d}_4$; Labelled 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 _in vivo_ studies in humans. 2-Ethylhexanol with two deuterium atoms in the α-position was synthesized as outlined in the renewal proposal (October 1973). Accordingly, commercial 2-ethylhexanol was oxidized with chromic anhydride in pyridine to afford 2-ethylhexanoic 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-ethylhexanoate with lithium aluminum deuteride in ether at room temperature and quenching of the reaction mixture with water gave crude 2-ethylhexanol-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-ethylhexanol-α-$\underline{d}_2$ was converted to the correspondingly labelled bis(2-ethylhexyl) phthalate-$\underline{d}_4$ (DEHP-(R)$\underline{d}_4$) by reaction with a 0.5 molar equivalent of phthaloyl chloride in tetrahydrofurane containing one equivalent 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 coinjuction with an unlabelled sample.

2. Purification of Butyl Carbobutoxymethyl Phthalate:

Commercial samples of butyl carbobutoxymethyl phthalate were foundto 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 carbobutoxymethyl 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 carbobutoxymethyl phthalate to afford dibutyl phthalate occurs upon standing (probably *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°).

Effects of BCBP on Blood Coagulation in Vitro

BCBP 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: BCBP 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 ctonrol 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 and 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 0.025 mg/ml and collagen 0.1 mg/ml.

The addition of BCBP either at the 0.1 or 0.01mM level did not appear to affect the clotting parameters studied with the exception of the platelet studies. No significant difference was observed between the control values using a methanol or plasma blank and the samples containing the plasticizer.

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 0.01mM 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 I 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

John A. Penner, M.D.
Professor
Internal Medicine
Jan. 11, 1974

MONITORING OF HUMAN TISSUES FOR THE PRESENCE OF PLASTICIZERS

From July 1973 until the present, various tissues have been obtained from 25 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, 141 separate tissue specimens have been obtained. Of this number 48 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.

RESULTS TO DATE

Refer to Table II for the quantitative tissue assay data for DEHP

TENTATIVE CONCLUSIONS UTILIZING AVAILABLE DATA

(1) The liver appears to contain the highest 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 data would seem to contradict those of Jaeger and Rubin (New England Journal of Medicine 287:1114, 1972) who found large amounts of DEHP in lung, particulary in cardiopulmonary bypass patients.

(2) The spleen also consistently contains DEHP, often in trace amounts. This finding may reflect the participation of the organ in the reticulo-endothelial system.

(3) One half the heart specimens analyzed contained DEHP, often in substantial amounts. High concentrations of DEHP have been reported in the lipid fractions of beef heart mitochondria which may account for the concentration in human heart.

(4) Of eight lung specimens analyzed, five did not contain detectable DEHP and the remainder contained only a trace of the chemical.

(5) The greatest single concentration of DEHP was found in the liver of a patient with no known recent history of transfusion (A-53).

(6) The patient with the greatest amount of DEHP in multiple organs was a 90 year old woman who died in a mental institution without a history of blood transfusion (YA-89). Necropsy A-90, a 25 year old woman who received multiple transfusions, had quantities of DEHP present of a similar magnitude.

(7) The one patient who underwent chronic renal dialysis for two years with the attendant high exposure to plastic material had less DEHP detectable than any other patient (A-16). Similarly, patient A-107 was a plasmapheresis donor for one year and had extensive exposure to plastic blood bags without marked DEHP accumulation.

(8) Patient A-92, an infant who survived only two days, had no transfusion of blood or blood products and yet had a substantial quantity of DEHP in the liver. This finding suggests exposure and subsequent accumulation in-utero. Rapid accumulation from a source other than blood during the two days of life spent in the hospital can not be excluded.

(9) One last interesting observation is that both patients A-15 and A-192 had high concentrations of DEHP in liver and heart. The only other patient with comparable levels has been discussed above (YA-89). It may be coincidental, but the former two patients both died in house fires.

The above conclusions are tentative due to limited data. Final correlation must await further tissue analysis.

TABLE II

μg/g Wet Tissue

Necropsy No.	Age and Sex	Clinical Diagnosis	Transfusion and Hemodialysis History	Liver	Omentum	Spleen	Heart	Lung	Kidney	Brain
A-15	44M	90% whole body burn	7 units whole blood, 10 units plasma	.0227	TBA	0	.0218	0	TBA	NA
A-16	50M	Acute rejection cadaver renal transplant	5 units whole blood, 2 years hemodialysis thrice weekly	Trace	TBA	0	0	0	0	NA
A-23	41F	Acute myocardial infarction	None known	0	0	Trace	.0063	Trace	Trace	NA
A-52	40F	Chronic granulocytic leukemia	16 units platelets, 3 units RBC's	TBA	TBA	.0223	0	TBA	TBA	NA
A-53	64M	Bronchogenic carcinoma	None known	.0727	TBA	Trace	0	0	0	NA
A-61	4wkM	Multiple congenital anomalies	20 ml. RBC's	TBA	NA	TBA	NA	NA	NA	NA
YA-89	90F	Arteriosclerotic heart disease	None known	.0157	TBA	Trace	.0222	0	.0162	NA
A-90	25F	Gram negative shock post partum	22 units platelets, 23 units RBC's, 6 units plasma, 3 units blood	.0176	TBA	Trace	NA	NA	.0130	NA
A-92	2daysM	Potter's syndrome with 69 chromosomes	None known	.0151	NA	NA	NA	NA	NA	NA
A-98	53F	Idiopathic cardiomyopathy	None known	TBA	NA	.0053	0	TBA	.0069	NA
A-107	26M	Wolf-Parkinson-White Syndrome	None known. Plasmapheresis donor for 1 year	.0104	TBA	0	NA	TBA	.0053	TBA
A-163	53M	Chronic granulocytic leukemia	9 units RBC's, 5 units platelets	.0358	TBA	.0047	TBA	Trace	Trace	TBA
A-164	52M	Bronchogenic carcinoma	None known	TBA	TBA	TBA	TBA	Trace	0	Trace
A-192	20M	Carbon monoxide poisoning in house fire	None known	.0267	TBA	TBA	.0155	0	TBA	TBA

TBA - To be analyzed
NA - Tissue not available
Trace - Detectable trace which could not be calculated

MASS SPECTRAL IDENTIFICATION OF COMPOUNDS LEACHABLE FROM PLASTIC MEDICAL DEVICES

The following is an abstract of a paper to be presented at the Sixth Annual Central Regional Meeting of the American Chemical Society, April 21-24, in Detroit, Michigan. Study of the individual tubing sets is nearly complete, and study of the tubing/kidney combinations is in progress.

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 plasticisers, such as di-(2-ethylhexyl) 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-ethylhexyl) adipate (DEHA) and butyl carbobutoxy 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.

PLASMA EXTRACTION OF PLASTICIZERS FROM "MEDICAL GRADE" POLYVINYLCHLORIDE TUBING

Ronald E. Easterling, Elvin Johnson and E. A. Napier, Jr.

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

RUNNING TITLE: Plasticizers from Polyvinylchloride Tubing

Up to 2 mg of Di-2-ethylhexyl phthalate (DEHP) have been extracted in 5 hours from polyvinylchloride 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° 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 (4) computerized gas liquid chromatographic-mass spectrometer.

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

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 when the recirculation was turned off. From 8.9 to 13.2 mg of DEHP were extracted form the 4 sets of tubing within six hours.

Diethyl-hexyl-adiptate (DEHA) was found 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 specimen, 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 experiments 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 isolated liver preparation (1). These reports indicate the need for controlled studies with other conduit materials (such as teflon or silicone rubber) when PVC tubing is used to perfuse isolated organs.

The significance of phthalate extracted into protein containing solutions for the intact organism 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 their 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 have 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 R.J. Rubin. Environmental Health Perspectives. DHEW Publication No. (NIH) 72-318, Research Triangle Park, North Carolina. January 1973, pp. 95-102.

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

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

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

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

6. Jaeger, R.J. and R.J. Rubin. New Eng. J. Med. 287, 1114, (1972).

7. Schreiner, G.E. Trans. Am. Soc. Artif. Int. Organs, 16, 544 (1970).

8. Burton, B.T., K.K. Krueger and F.A. Bryan, Jr. JAMA 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

Abstract

Di-2-ethylhexyl phthalate and butyl carbobutoxymethyl 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. Toxic levels for di-2-ethylhexyl phthalate are approximately one half as great as the levels found in blood which has been stored in polyvinyl chloride blood bags for 21 days.

Report

Phthalates, which are used as plasticizers for many polyvinyl chloride (PVC) items in medical and laboratory use, have recently come under closer investigation (1). 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 (2, 4), and in anticoagulant solution stored in PVC bags (2, 3). Also, DEHP has been found in the tissues of deceased patients who had recently received transfusions (4). Certain formulations of PVC tubing used in hemodialysis units have been shown to release DEHP into circulating plasma (5). Other tubing formulations have revealed the presence of another plasticizer, butyl carbobutoxymethyl phthalate (also known as butyl glycolyl butyl phthalate, or BGBP) (5, 6). Although phthalates are the principle additive to PVC (up to 40% by weight), stabilizers and organotin compounds are also present in small amounts, and also can be leached from PVC (7). Although several studies have been conducted on these compounds, more detailed analyses of the present and future effects of phthalates are warranted.

In studies on rats, both DEHP and BGBP have been shown to have a low acute toxicity(8, 9). Moreover, both compounds induce some teratogenic effects when administered to rats in very large doses (8). Since in vivo studies are difficult to interpret and reflect complex interactions, it is useful to study the subacute, or subtle, toxicity of phthalates by tissue culture assay. We have conducted experiments using the direct addition of DEHP and BGBP to the culture medium. Similar methods have

been used to study the effects of DEHP on L-929 mouse fibroblasts (1), although the procedures for these studies have not been published. While previous *in vitro* studies have described an agar overlay method (10), 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 permeate the agar layer. One study using this method noted that the most soluble phthalates were the most toxic (9), while no toxicity was reported for DEHP.

We have chosen to use the human diploid cell strain WI-38 which is a useful study tool due to its diploid nature and its rapid generation time (11). The WI-38 cells were grown in Basal Medium Eagle (Gibco G-13) with 10% calf serum (Flow Labs). The cultures used for protein determination (Fig. 2) were grown in Falcon T-25 flasks 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 (12). Cells grown on cover slips were stained with oil red O and hematoxylin (Fig. 1).

In that DEHP solubilizes more readily in the lipoprotein fraction of plasma (5), both of the 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 solubilized, and were visible as an emulsion. To determine the true amount in solution, the emulsion was removed by centrifugation and the aqueous samples were extracted by the Folch technique (13). Quantitation of these samples was performed by Gas Liquid Chromatography (GLC) using a 1% SE30 column

(1.8 m. by 4 mm.) on 80-100 mesh Chromosorb-W, at 200° C. Assays were also conducted on a computerized GLC mass spectrometer (14).

GLC analysis reveals that the addition of 0.25 mM, 0.50 mM, and 1.00 mM BGBP to the culture medium results in soluble concentrations of 7 μM, 10 μM, and 14 μM respectively. Cells treated with 14 μM BGBP (Fig. 1b) for 6 days show a decreased cell density and a thinning cytoplasm when compared to controls (Fig. 1a). Total cell protein is also affected by BGBP (Fig. 2a). From this data it is possible to determine an ID_{50} value (dose which causes 50% growth inhibition in tissue culture) of 12 μM for BGBP on WI-38 cells. Separate experiments have shown that a concentration of 18 μM BGBP will kill the entire cell population after 6 days in culture.

Concentrations of 0.25 mM, 0.50 mM, and 1.00 mM DEHP added to the culture medium result in soluble concentrations of 51 μM, 69 μM, and 160 μM respectively. The appearance of cells treated with 160 μM DEHP for 6 days is very similar to that shown by cells treated with 14 μM BGBP. In addition to a noticeable growth inhibition, the cytoplasm is more firmly attached to the slide in the DEHP treated cells. This fact is further demonstrated by the increased resistance of DEHP treated cells to trypsinization with 0.25% trypsin, 0.1% EDTA solution. The flasks treated with 51 μM and 69 μM DEHP show decreased cell protein (Fig. 2b), and a longer generation time, although these cells were still viable at the end of 9 days of treatment. The cells treated with 160 μM DEHP exhibit an even greater protein decrease; degenerate by day 7, and die by day 9. The ID_{50} value

for DEHP on WI-38 cells is approximately 70 μM. A value of 50 μM has been reported by Autian's Laboratory (1) for mouse L-929 cells. With both DEHP and BGBP treated cells, as well as with the controls, there is an initial increase in growth rate after refeeding (Figs. 2a and b). This is probably due to the growth stimulatory effect of fresh calf serum and to nutrient addition, which temporarily override the effects of added phthalate.

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 cell concentrations there is very little cell division occurring. While these dense cell 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.

Dillingham and Autian (15) found that nonreplicating L-929 cells are less affected than replicating ones when treated with dimethoxyethyl phthalate. This may be linked to the findings by Warren and Glick (16) 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 due to mitosis, which creates membrane instability and a higher susceptibility to toxicants at this stage. Studies of the effects of dimethoxyethyl phthalate, dimethyl phthalate, and diethyl phthalate (15, 17) on replicating L-929 cells in culture indicate an increased protein turnover rate with increased

concentrations of these compounds. In addition, the authors note that there is a suppression of new protein synthesis where phthalate exceeds certain concentrations.

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 levels for 3 and 5 days did not show decreased viability or a change in membrane attachment after subculture.

Aside from the reversibility studies, our data demonstrates that BGBP (M.W. 336) is more toxic than DEHP (M.W. 391). Except for dimethoxyethyl phthalate (DMEP) (M.W. 282), previous studies conducted *in vitro* and *in vivo* have revealed a direct correlation between molecular weight and phthalate toxicity (1). The only other exception to this rule is the reported increased toxicity of dibutyl phthalate (DBP) (M.W. 278) *in vivo* (8, 9). While DEHP has the higher molecular weight, it may be that the butyl group (as in DBP) could be responsible for its increased toxicity. Preliminary studies in our laboratory indicate that dioctyl phthalate (M.W. 391) has the same toxicity as DEHP while DBP is

less toxic than DEHP, but more readily leached from plastic surfaces. We are currently evaluating the toxicity of an antioxidant (2,6-di-ter-butyl cresol) and a nonphthalate plasticer (di-2-ethylhexyl adipate) which we have recently detected in plastic medical devices and which are leachable from the plastic.

The fate of DEHP and BGBP in tissues is not fully understood. Jaeger and Rubin found that DEHP does not appear to be metabolized in an isolated rat liver perfusion system (5), while BGBP is metabolized to glycolyl phthalate. More recent evidence has shown that phthalic acid and phthalic acid containing metabolites of DEHP can be found in the urine of certain human patients who have received numerous units of stored blood (4) and in the urine of rats fed DEHP orally (19). There is also evidence that DEHP does not appear to be easily metabolized, and can accumulate in human tissue (4), fish tissue (20), and in bovine heart tissue (21). Studies of the absorption and metabolism of related compounds, 2-alkylalkanoates, in rats (22), 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. To date, there is no evidence of BGBP accumulation in 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 (4). Equally important, 486 μM DEHP has been detected in platelet concentrates stored at 22° C for only 2 days (18). 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. Although there does not appear to be an immediate clinical problem, evidence of subtle toxicity from phthalate esters found in PVC contaminated plastics and possible tissue accumulation of DEHP warrants serious concern. Since nearly one billion pounds of phthalates are produced annually in the U.S. (23), further efforts to find nontoxic plasticizers for medical devices and to restrict the dumping of phthalates as industrial wastes should be considered.

Adelaide E. Jones
Raymond H. Kahn
Department of Anatomy
The University of Michigan
Ann Arbor, Michigan 48104

Edward A. Napier, Jr.
Departments of Biological Chemistry and Internal Medicine
The University of Michigan
Ann Arbor, Michigan 48104

REFERENCES

1. J. Autian, Environmental Health Perspectives, Exper. Issue 4, June 1973, p. 3. J. L. Marx, Science 178:46 (1972).
2. R. J. Jaeger and R. J. Rubin, Lancet: 151 (July 18, 1970).
3. W. L. Guess, J. Jacob and J. Autian, Drug Intel. 1:120 (1967).
4. R. J. Jaeger and R. J. Rubin, The New Eng. J. Med. 287:114 (1972).
5. R. J. Jaeger and R. J. Rubin, Science 170:460 (1970).
6. A. S. Trimble, B. S. Goldman, J. K. Yao, L. K. Kovats, W. G. Bigelow, Surgery 59:857 (1966).
7. B. Braun, and H. J. Kummell, Deut. Apoth. Ztg. 103:467 (1963).
8. A. R. Singh, W. H. Lawrence, and J. Autian, J. Pharm. Sci. 61:51 (1972).
9. D. Calley, J. Autian, and W. L. Guess, J. Pharm. Sci. 55:158 (1966).
10. W. L. Guess, S. A. Rosenbluth, B. Schmidt, J. Autian, J. Pharm. Sci. 54:1545 (1965).

    S. A. Rosenbluth, G. R. Weddington, W. L. Guess, J. Autian, J. Pharm. Sci. 54:157 (1965).

    J. Nematollahi, W. L. Guess, and J. Autian, J. Pharm. Sci. 56:1446 (1967).
11. L. Hayflick, Exp. Cell Res., 25:585 (1961).
12. V. I. Oyama and H. Eagle, Proc. Soc. Exp. Biol. Med. 91:305 (1956).

13. J. Folch, M. Lees, and G. H. Stanley Sloane, J. Biol. Chem. 226:497 (1957).

14. C. C. Sweeley, M. I. Krichevsky, B. D. Ray, W. I. Wood, and J. F. Holland, Analyt. Chem. 42:1505 (1970).

15. E. O. Dillingham and J. Autian, Environmental Health Perspectives, Exper. Issue no. 3, Jan., 1973, p. 81.

16. L. Warren and M. C. Glick, J. Cell Biol. 37:729 (1968).

17. E. O. Dillingham, Ching-Hsien Wu, J. Autian, Toxicol. Appl. Pharmacol. 22:318 (1972).

18. R. J. Jaeger and R. J. Rubin, Transfusion 13:107 (1973).

19. P. W. Albro, R. Thomas and L. Fishbein, J. Chromatogr., 76:321 (1973).

20. F. L. Mayer, D. L. Stalling, and J. L. Johnson, Report, Fish-Pesticide Research Laboratory, Bureau of Sport Fisheries and Wildlife, U. S. Dept. Inter., Columbia, Mo., 1971.

21. D. J. Nazir, M. Beroza and P. P. Nair, Fed. Proc. 26:412 (1967).

22. T. A. Saladin, and E. A. Napier, Jr., J. Lipid Res. 8:342 (1967).

23. A. Hall, Mod. Plast. 48:58 (1971).

24. This research was supported by a contract with the National Heart and Lung Institute, NIH-NHLI-73-2936-B, and, in part, by a Research Cancer Development Award NHI 1-K4-HE-38,880, and the Michigan State Mass Spectrometry Facility (NIH RR00480).

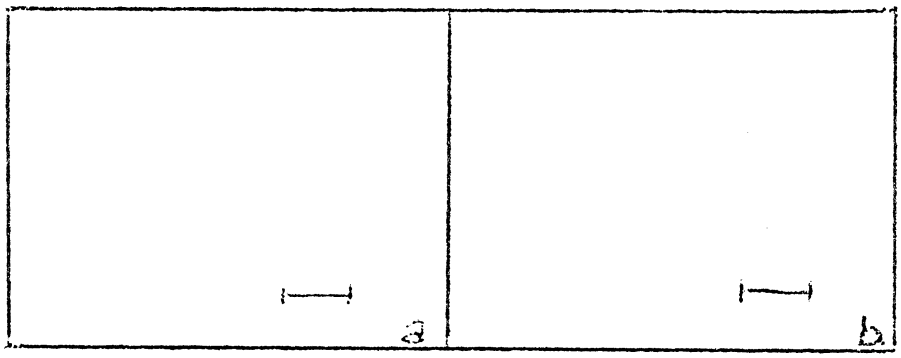


Fig. 1. Monolayer culture of WI-38 cells exposed to 14 μM BGBP for 6 days (b) as compared to the effect of the control medium (a).

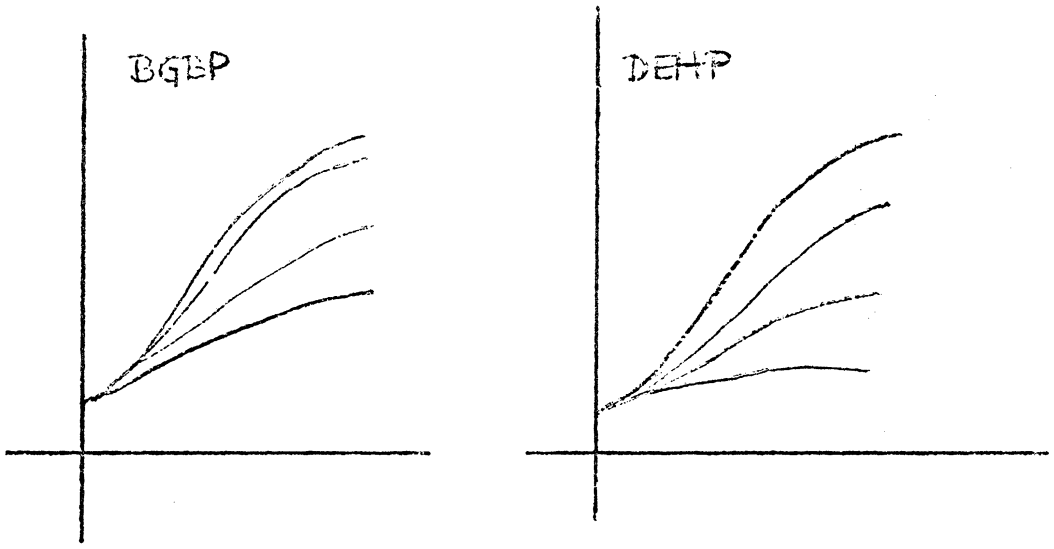
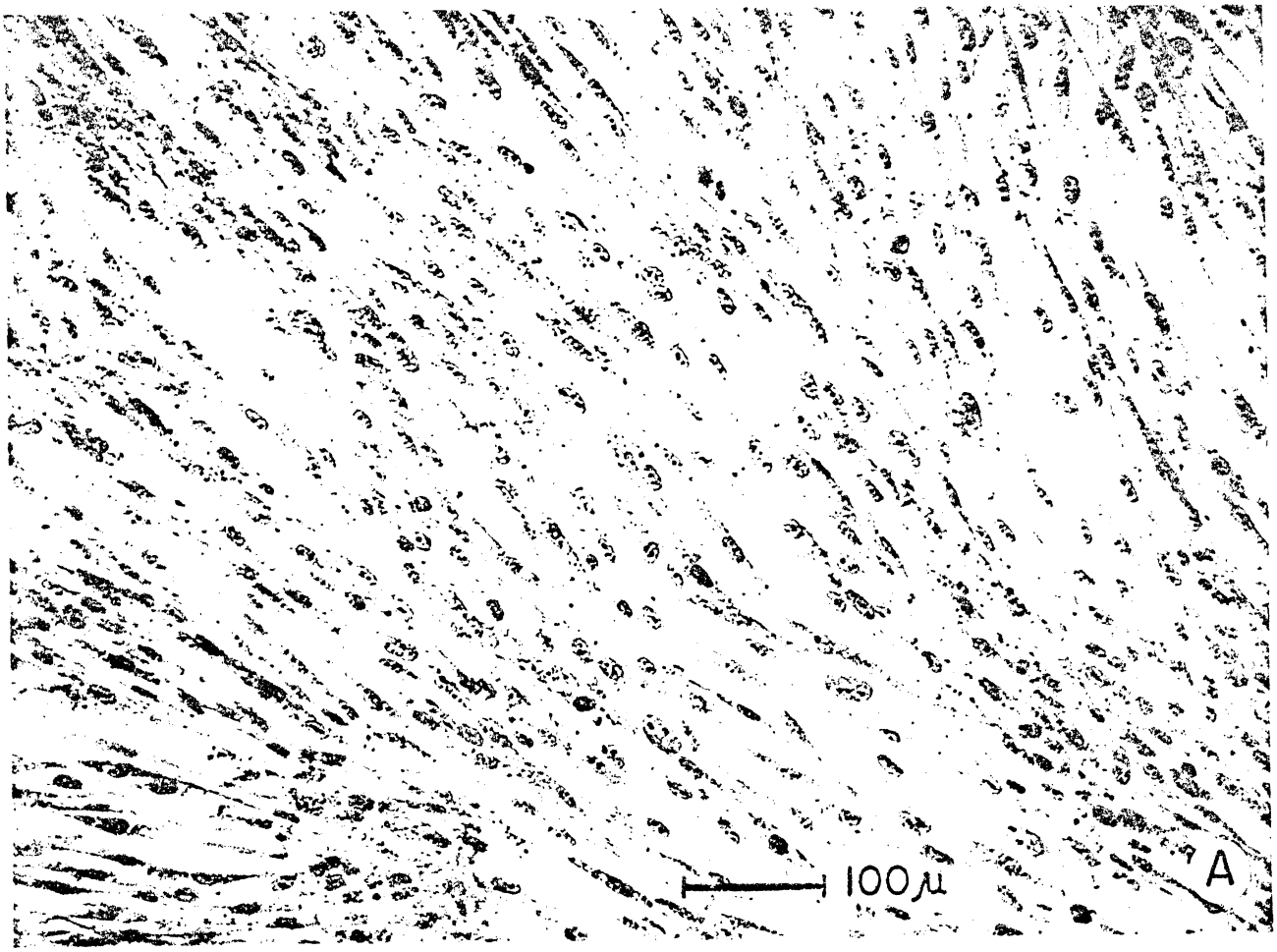
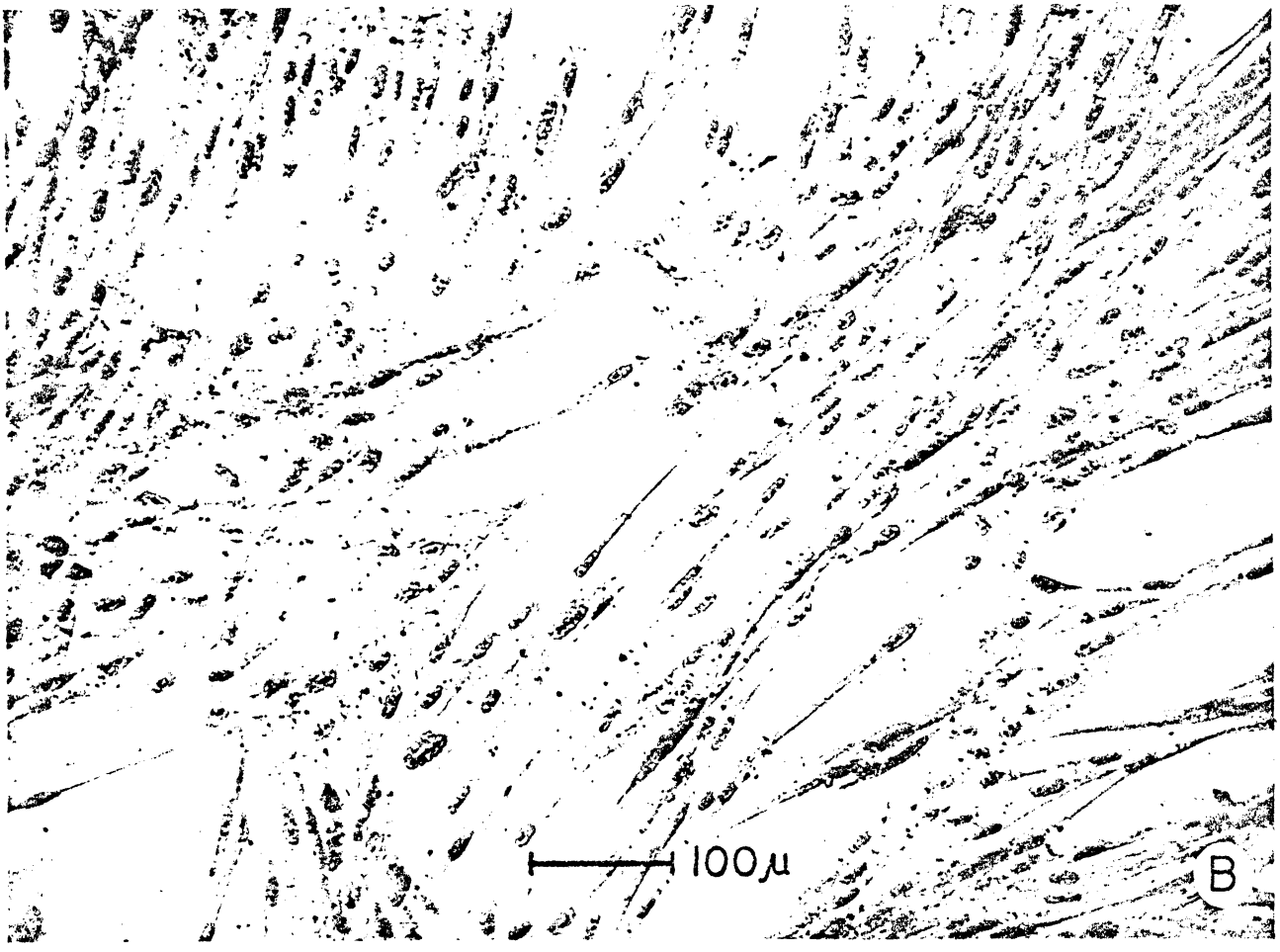
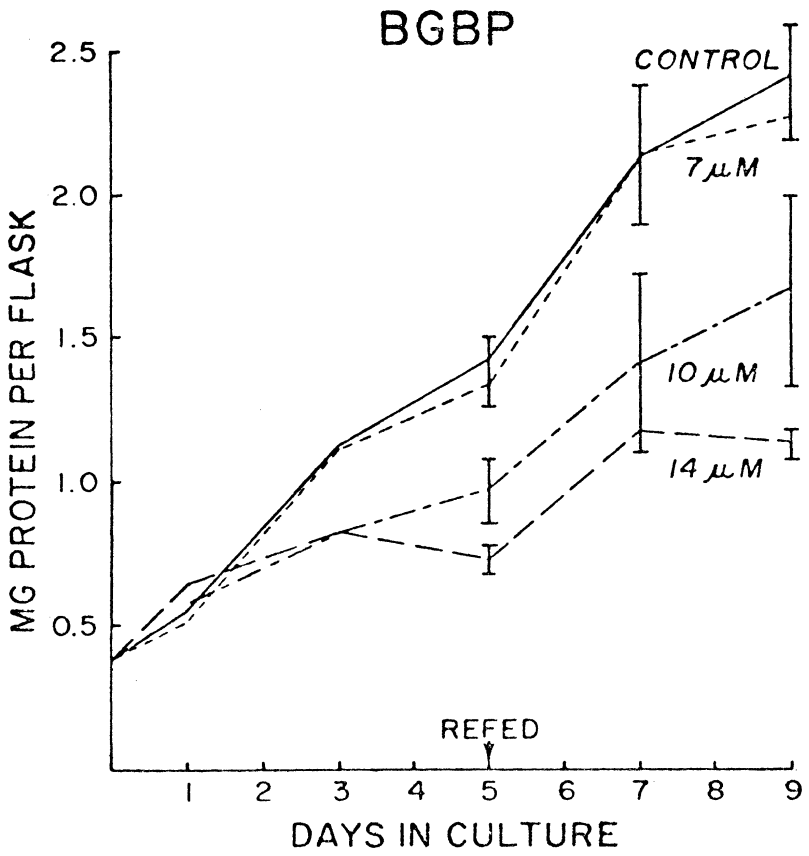
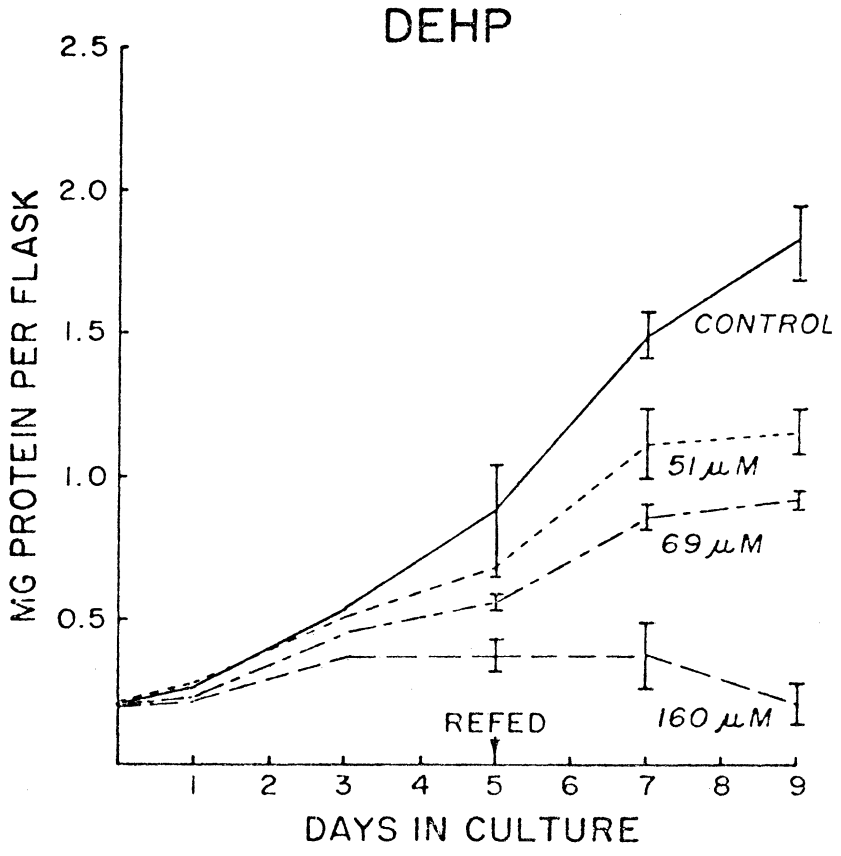


Fig. 2. Protein content of cells treated with BGBP (a) and DEHP (b). Each point represents the mean value for 3 sample flasks. Brackets indicate ± 1.5 standard error of the mean.


100μ
A


100μ
B


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


DEHP
CONTROL
51 μM
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