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STUDIES ON THE EXTRACTION, ACCUMULATION AND METABOLISM OF PHTHALATE ESTER PLASTICIZERS FROM POLYVINYL CHLORIDE MEDICAL DEVICES

BY

Rudolph John Jaeger

DISSERTATION

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of The Johns Hopkins University in conformity

with the requirements for the degree of

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ABSTRACT

Evidence is presented which demonstrates that phthalate-ester plasticizers are extracted from polyvinyl chloride medical devices when blood or protein containing solutions are stored or circulated in them. This extraction of plasticizers is a consequence of the protein and/or the lipid content of such fluids and does not occur with saline solutions.

The administration of one plasticizer, butyl glycolylbutyl phthalate, in both the intact rat (ip) and isolated, perfused rat liver (iv) results in the appearance of glycolyl phthalate. The exact structure of this metabolite is demonstrated through the use of analytical techniques. A second plasticizer, di-2-ethylhexyl phthalate, was found to be extracted from PVC plastic tubing, but its metabolism to phthalic acid could not be demonstrated in the rat. Accumulation of this plasticizer by tissues was seen to occur in the rat when the plasticizer was given by an intravenous route, and specifically, concentration occurred in the lung, liver and spleen of the intact rat, as well as in the isolated perfused rat liver.

Tissues from some humans who had been exposed to di-2-ethylhexyl phthalate-contaminated blood were found to contain this plasticizer. Not all cases were positive for this material, and metabolism of this compound in the human could be shown when the methods of Shaffer et al (1945) were employed. Further, it was observed that in urine from normal human subjects, a phthalic acid containing material could be found after hydrolysis, and thus, a possible source of some phthalate compound as an environmental (dietary) contaminant was suspected.

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Rats and mice were found to have enhanced hexobarbital sleeping times after the intraperitoneal injection of the plasticizer di-2-ethylhexyl phthalate (500 mg/kg). Further, decrements in behavior, losses in body weight, and decreased food and water consumption were also observed. It was shown that the material was an irritant to the peritoneal cavity, and thus, some of its effects on pharmacologic and behavioral responses could be ascribed to some stress mediated effect. It was further observed that intravenous injection of large doses of the plasticizer di-2-ethylhexyl phthalate resulted in a highly significant loss in body weight.

Polyvinyl chloride materials were found to cause platelet aggregation but this could be altered by prior treatment of the blood storage bag with a wall bonded heparin coating. Another plastic material which contains no plasticizer was not found to alter the degree of platelet aggregation with storage.

Isolated units of platelet rich plasma were found to contain high concentrations of the plasticizer di-2-ethylhexyl phthalate after only two days of storage. The concentration of the plasticizer recovered from the packed platelet fraction was found to be two times greater than that found in the surrounding plasma. A role of this phthalate ester in reducing the platelet viability of stored human platelet concentrates may be possible.

Experiments on isolated beating heart cells in tissue culture indicated that both di-2-ethylhexyl phthalate and di-2-ethylhexyl adipate were toxic to these cells. The level of di-2-ethylhexyl phthalate which

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was toxic to the heart cells was also a concentration of the plasticizer which could be found in units of one day old, plastic bag-stored, human blood.

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LIST OF ABBREVIATIONS

DEHP	di-2-ethylhexyl phthalate
BGBP	butyl glycolylbutyl phthalate
DEHA	di-2-ethylhexyl adipate
GP	glycolyl phthalate
PA	phthalic acid
2-EH	2-ethylhexanol
PVC	polyvinyl chloride
PVC-GBH	polyvinyl chloride with graphite bonded heparin
TMS	trimethyl silane
PCA	perchloric acid
ACD	anti-coagulant citrate dextrose
BSA	bovine serum albumin
CPB	cardio-pulmonary bypass
HD	hemodialysis
SFP	screen filtration pressure
nm	namometer

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I. INTRODUCTION

An increasingly complex technology has been developed by man in an attempt to alter and enhance his environment. This technological advance has not been without cost, and the present ecosystem, while more productive and fertile, has become polluted by previously unknown chemical compounds. These materials, both intentionally and unintentionally, contaminate the food we eat, the clothes we wear, the water we drink, and the air we breathe. It is highly unlikely that an individual who lives in modern society will not be exposed to numerous foreign chemicals. Subsequent to such exposure, the normal mechanisms of metabolism, storage, and excretion will be brought to bear. These responses are an attempt by the organism to adapt, and the final balance of the species with its ecosystem will be the sum of all adaptive changes. However, adaptation like technology is not without cost, and the question of the possible limitations of such responses must be considered.

To exemplify the kinds of contamination that are found today, the following examples and their consequences are given: 1. pesticide residues in food may cause alterations in the activity of certain enzymes needed for neurohumoral transmission (Murphy and Cheever, 1968) and may alter hepatic metabolism of foreign chemicals, such as drugs (Conney, 1967 ; Ecobichon, 1970). 2. heavy metals such as mercury have been found in fish and certain meats and their presence in the diet may lead to teratogenesis and CNS dysfunction (Matsumoto, et al, 1965).

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3. potent carcinogens are present in cigarette smoke (U.S.P.H.S., 1964) and in incomplete combustion residues of fossil fuels (Stern, 1968). These agents may give rise to lung cancer as a result of inhalation over a long period of time (Arcos, et al, 1968). 4. NO_2, SO_2 and O_3 may result from the interaction of industrial and automotive exhaust gas with the atmosphere and sunlight (Coffin and Blommer, 1967). These pollutents have been documented to alter lung function (Boren, 1967; Jones, et al, 1970; Hurst, et al, 1970; Thomas and Rhoades, 1970; Dowell, et al, 1970; Tse and Bockman, 1970). The increased death rate after episodes of "killer smog" (Hodgson, 1970) is a phenomenon becoming more common as levels of gaseous pollution increase.

While it is not the author's intention to list all possible environmental contaminants and their detrimental effects, it can be seen from this partial listing that there is sufficient cause for alarm. The appearance in living organisms of any compound that is not normally present, is thus reason for concern and for further investigation of toxicologic implications.

This dissertation is a result of an initial observation, in this laboratory, that a biologically unknown material appeared in the perfusate of an isolated, perfused rat liver. Subsequent analysis disclosed that this material was an hepatic metabolite of a plasticizer which had been leeched from the plastic tubing of the perfusion apparatus by the circulating blood.

Based on this finding, the possibility arose that similar contamination of human blood in contact with a variety of plastic medical

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devices might occur. Polyvinyl chloride plastic materials are widely used in hospitals for hemodialysis, cardiac by-pass during open-heart surgery, and for the storage of blood. The contamination of blood and blood fractions stored in plastic medical devices will be reported, and the metabolism of these compounds by the human will be shown. The possible accumulation of plasticizers in body fat depots of certain selected individuals will be considered. The rat and the mouse will serve as models to document the biologic effects of phthalate esters. Biological effects more subtle than overt toxicity will be considered. This approach was considered to be necessary since it has been shown that esters of phthalic acid are generally regarded as safe for use in materials to which the human is exposed. From the animal model, the consequences of such exposure will be considered.

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II. HISTORICAL REVIEW

A. Polymeric Materials and Additives

Plastics represent a wide array of materials whose common chemical characteristic is that they are macromolecules that are formed by the polymerization of monmeric units. Their common physical characteristic is that they can be molded or formed into a variety of shapes. As plastics, they may be modified by the addition of a variety of chemical additives so that the finished product may be soft or rigid, colored or opaque, inextensible or extremely pliable. It is because of this variety of physical properties that synthetic plastic materials can be used in an enormous variety of applications. They have become so ubiquitous that there are very few aspects of existence into which plastic materials have not been introduced.

In general, plastics have been considered quite safe for use in situations where humans are exposed to them. This may be true of the pure polymer since it is known that such substances of high molecular weight are highly insoluble in aqueous media and thus are unlikely to reach a concentration in body tissues necessary to cause harm. However, this is not necessarily true for the additives of low molecular weight which are used to change and enhance the physical characteristics of the initial polymer.

The additives which are used in plastics may have several functions; they are: 1. contribution of a color to the final product (colorant),

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2. prevention of air oxidation (antioxidant), 3. stabilization of the polymer to the adverse effects of heat, light and radiation (stabilizer), and 4. changes in the crystalline characteristics and a reduction in the glass transition temperature (plasticizer). It is this last additive which is present in largest amounts. One type of plastic, polyvinyl chloride, requires the use of all four types of additives but the amounts of each present in the finished product are small relative to the plasticizer content. This additive may amount to 30 - 40% of the final product weight (Gulbring, 1964), depending on the degree of softness desired.

The material, polyvinyl chloride, is presently the favored material of manufacturers. Its properties may be altered so greatly by additives that it can be used in an almost limitless variety of ways. The plastic is formed from the end to end condensation of vinyl chloride (CH_2=CHCl), and leads to a rigid macromolecular polymer which is totally insoluble in water and most organic solvents. It is quite resistant to the action of most acids and bases, and is soluble in only a few cyclic ketones. The presence of a carbon to chlorine bond requires that a stabilizer be added to prevent the release of potentially toxic HCl.

Due to PVC's inherent rigidity, a plasticizer is needed to reduce the van der Waals interaction between neighboring polymer chains. Thus, by intercollating among the molecules, the plasticizer replaces the relatively strong polymer-polymer interaction with a much weaker polymer-plasticizer interaction. The degree of crystallinity of the polymer is thus reduced and the material is now quite pliable and has a high

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impact resistance.

Commonly encountered plasticizing agents are usually lipid soluble substances, in most cases esters of organic and inorganic acids. A number of commonly used acids are phosphoric, citric, adipic, and phthalic acids. Certain of these are approved by the Food and Drug Administration for use in food packaging materials and this list is shown in table one.

Due to certain physical characteristics and economic factors, the most popular plasticizers for use in PVC materials are esters of phthalic acid. The most common of these, in present usage, is di-2-ethylhexyl phthalate. Butyl glycolylbutyl phthalate is used to a lesser extent. The available toxicologic literature of these two additives is reviewed.

B. Di-2-ethylhexyl phthalate - Oral Toxicity

The acute oral toxicity of DEHP in rats first evaluated by Hodge (1943). At doses up to 34 grams/kg the only ill effect noted was diarrhea, this occuring primarily at the highest dose levels. Autopsy of the animals twenty-four hours after DEHP revealed no peritoneal abnormalities except for a marked enlargement of the stomach. This was attributed to overeating after the temporary starvation due to diarrhea.

Shaffer et al (1945) administered single acute doses to rats and rabbits. The LD_{50} was determined for the subsequent fourteen day period. Half of the animals died more than 6 days after dosing rather

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TABLE ONE

FOOD AND DRUG ADMINISTRATION APPROVED PLASTICIZERS FOR USE IN THE MANUFACTURE OF FOOD PACKAGING MATERIALS

SUBPART E - FOOD ADDITIVES

121.2001 (e) plasticizers

Acetyl tributyl citrate

Acetyl triethyl citrate

p-tert Butylphenyl salicylate

Butyl stearate

Butyl glycolylbutyl phthalate

Dibutyl sebacate

Di-2-ethylhexyl phthalate (for foods of high water content only)

Diethyl phthalate

Diisobutyl adipate

Disooctyl phthalate (for foods of high water content only)

Diphenyl-2-ethylhexyl phosphate

Exopidized soybean oil (Iodine number, maximum; and oxirane oxygen, minimum 6%)

Ethyl glycclylethyl phthalate

Glycerol monoleate

Monoisopropyl citrate

Mono, di, and tristearyl citrate

Triacetin (glycerol triacetate

3-(2-Xenoyl)-1, 2-expoxy propane

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than immediately. Thus a delayed toxicity was apparent. The LD_{50} for rats was 30.6 grams/kg and for rabbits was 33.6 grams/kg. Histologic examination of the tissue of animals that had died indicated a generalized cloudy swelling of the liver and a moderate degree of cloudy swelling of the kidneys with granular secretion in the tubules. Thus, damage to these organs contributed to the cause of death.

Shaffer et al (1945) also conducted subacute toxicity studies in rats over a ninety day period. The animals received the material in the diet at the levels of 1.9, 0.9, 0.4, and 0.2 grams/kg/day respectively. At the three highest dose levels, growth rates were slightly retarded. At the two highest dosages, tubular atrophy and testicular degeneration were noted.

Two adult male human subjects were given doses of 5 and 10 grams. At the higher dose, mild catharsis and gastric disturbance were noted while no overt symptoms were observed at the lower dose.

Carpenter, Weil, and Smyth (1953) studied the chronic toxicity of DEHP fed to rats for two years, to guinea pigs for one year and to dogs for one year. Rats were studied over two generations in an attempt to determine if chronic exposure to DEHP in the diet could affect fertility.

The study with rats disclosed no change in mortality or life expectancy. Body weight gain at a dietary level of 0.4% of the diet was reduced in both generations of male rats. In the males of the parental generation and in all rats of the first filial generation at the 0.4% dose

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level, liver and kidneys weights were elevated over controls when expressed as a percentage of body weight. No other adverse symptoms were reported in numbers of neoplasia, hematology, or fertility.

Guinea pigs were fed 0.13 and 0.04% DEHP in the diet. In these animals, the life expectancy of experimental groups was significantly greater for treated animals than for controls. The females at both DEHP doses had higher liver and kidney weights than controls or males of their own groups. No abnormal micropathology or neoplasms were associated with the DEHP regimen.

Dogs received doses of DEHP of 0.06 ml/kg/day for one year. No effects on liver and kidney weight or tissue microphatology were noted. This dose was considered a no effect level for DEHP in this species.

Harris et al (1956) studied the effect of chronic feeding of DEHP in rats and dogs. The animals received the chemical at the levels of 0% (control), 0.1% and 0.5% in the diet (rats) while the two dogs studied received different doses (5 gms/kg/day by stomach tube or 0.1 gm/kg/day in the diet). The results of the study with rats indicated no alteration in mortality at either dose level, while food consumption and body weight were lower on the 0.5% regimen. Enlargement of the liver and kidney were seen at 3 and 6 months but no evidence of pathology could be detected.

The results of the dog study (14 weeks) indicates that no effects could be ascribed to DEHP except that the animal at the highest dose lost weight during the study. Data on liver and kidney weights were

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not recorded.

As a result of these studies, di-2-ethylhexyl phthalate has been judged non-toxic when administered orally. The conclusion of Harris et al (1956) may be used to sum up the available toxicologic literature on the oral toxicity of DEHP: "The minute traces that may appear in non-fatty foods wrapped in plastics in which (DEHP) is the plasticizer, on the basis of animal studies, pose no threat of toxic hazard".

C. Di-2-ethylhexyl Phthalate - Inhalation Toxicity

Shaffer et al (1945) examined the toxicity which resulted from the exposure of rats to a saturated mist of DEHP. This mist was produced by passing air through a wash bottle of heated plasticizer (170°C), followed by cooling to room temperature. The experiment was designed presumably to simulate the exposure of workers who might be involved in the manufacture of PVC. It was found that rats exposed under such conditions could survive for 2 hours and all the animals were dead after four hours. Under similar circumstances, glycerol was toxic after 30 minutes and fatal to all animals after 1 hour. Therefore, the toxicity of DEHP is less than glycerol when exposure is by the route indicated, and the authors concluded that inhalation of DEHP vapors does not represent a hazard significantly greater than that of other commonly encountered industrial chemicals.

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D. Di-2-ethylhexyl Phthalate - Intraperitoneal Toxicity

The toxicity of this compound was studied by Hodge (1943) in both rats and mice. He was unable to kill rats with doses up to 24 grams/kg. The only evidence of toxicity was diarrhea, and on autopsy, he observed that the peritoneal cavities were filled with a milky, watery emulsion, and the livers were enlarged. When animals were killed serially (1, 5, and 7 days) after the dose, he found that liver weights returned to normal. Histologic examination of liver biopsies from the treated rats indicated swelling of the cytoplasm, marked granularity, and moderate degeneration. Vacuolated areas were found which did not stain with either hematoxylin or eosin.

Similar findings were observed in mice, and at a maximal dose of 128 grams/kg, only 5% of the mice had died. Thus, an LD_{50} value could not be determined.

Shaffer et al (1945) were able to obtain an LD_{50} for DEHP in rats by the intraperitoneal route. This value, 30.7 grams/kg, was indentical to the value obtained after oral administration. They confirmed the observation of Hodge (1943) that the peritoneal cavity of treated rats contained a milky emulsion,and they reported that chemical tests indicated that this fluid contained phthalates. It was stated that absorption of DEHP from the peritoneal cavity was no more complete than from the digestive tract.

Studies by Mallette and Von Haam (1952) indicated that DEHP is of moderate toxicity when administered ip to rats. They found that

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doses greater than 2 gms/kg were uniformly fatal, and at lower doses, weight loss, leucocytosis, severe anemia, and hematuria were observed. The animals that survived the initial dose recovered after a period of one to two months and did not suffer permanent injury.

The acute intraperitoneal toxicity of DEHP in mice was also evaluated by Calley et al (1966). They found that this material given ip as an aqueous emulsion lead to an LD_{50} of 14.2 grams/kg. Doses of 500 mg/kg given by this route altered the hexobarbital sleeping time in that the amount of time that animals slept was reduced.

A series of studies on the subacute effects of DEHP in mice over a period of six weeks led to the conclusion that the compound, DEHP, depressed the rate of weight gain. All animals reached the same weight by the end of the period while the controls reached this level after three weeks. Animals who received DEHP had an extreme degree of peritonitis with adhesion formation, hepatic and testicular abscesses. The reason for the decreased rate of weight gain may have been a decreased food consumption due to severe abdominal irritation.

Organ to body weight ratios of the animals receiving DEHP indicated that liver values were increased while the testes value was found to decrease. Heart, lung, kidney, and spleen ratios were unaltered. No alterations in hematology were observed. The peritoneal abscesses and cloudy swelling were taken as evidence of pathology and control animals were normal in this regard. The irritant effect of DEHP was considered equal to daily injections of an equal volume of 20% ethanol.

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E. Di-2-ethylhexyl Phthalate - Intravenous Toxicity

Calley et al (1966) studied the effects in the rabbit of DEHP given intravenously as an aqueous emulsion. Dose of 50 mgs/kg were given repeatedly and the total dose administered was the sum of these injections. Blood pressure, EEG, EKG, and respiration rate were measured.

Each dose of DEHP had an effect on respiration rate, such that the rate was increased. However, the effect disappeared after five minutes at which time the next dose was given. No effects were seen on blood pressure until a minimum total dose of 350 mgs/kg was given. At this time, blood pressure decreased, but no changes were observed in the EKG or vector analysis. The authors ascribe this change in blood pressure as a vascular response to toxicity.

F. Di-2-ethylhexyl Phthalate - Miscellaneous Toxicities

Shaffer et al (1945) evaluated the toxicity of DEHP following absorption from the skin in rabbits. They found that the lethal dose was approximately 25 ml/kg. However, the authors stated that application of the chemical to the skin was not a reliable route, and the value obtained was an estimate. No skin injury resulted in this test.

These authors also tested the irritant action of DEHP by direct application of the undiluted material to the cornea of rabbits. No necrosis was observable by fluorescein staining and only a transient congestion of the eye lids occurred. Application of the material to the skin of

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humans had no effect acutely over a period of 17 days. No sensitization was noted.

Mallette and Von Haam (1952) tested DEHP on the skin of rabbits and reported that the material was moderately irritating. No sensitization was observed. On human skin, the material at a 10% concentration in propylene gycol was non-irritating to 87% of the subjects tested and caused a slight reaction in the remaining 13%. Three per cent of the individuals became sensitized. They concluded that the material is a moderate irritant and is only slightly sensitizing.

Calley et al (1966) using the dye extravasation test determined the irritant action of DEHP in rabbits. An aqueous suspension of the material was injected subcutaneously, and 15 minutes later, trypan blue was injected systemically. DEHP was maximally irritating 10 to 20 minutes after injection of the dye. The degree of irritation was comparable to equal volume of 20% ethyl alcohol.

Calley et al (1966) also evaluated the toxicity of DEHP on cells in tissue culture. At a dose of 50 mgs/ml, the compound had no grossly observable effect on chick embryo cells or mouse fibroblasts.

Guess and Haberman (1967) in their study of the toxicity of various plastics and plastic additives confirmed the findings of Calley et al (1966) that DEHP has no effect on chick embryo cells or mouse fibroblasts in tissue culture. They also tested the material in amnion cells and nasopharyngeal cancer cells in tissue culture. As with the previous cell lines, DEHP was found to have no grossly observable toxic

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effects at the concentrations studied.

McLaughlin et al (1963) reported on the toxic effects of a variety of chemicals introduced directly into the yolk sac of fertile eggs prior to incubation and hatching. They found that DEHP (0.05 ml undiluted) resulted in a 95% hatch relative to uninnolculated eggs, and an intermediate order of toxicity. The toxicity was similar in magnitude to an equal volume of ethyl alcohol.

Most recently, Bower, Haberman and Minton (1970) studied the effects of phthalate esters in the fertilized egg. They found that injection of 0.1 ml of undiluted plasticizer was without noticable effect on the number of eggs that hatched or on the number of malformations. They concluded that DEHP was without effect in this test system.

G. Summary of Di-2-ethylhexyl Phthalate Toxicity

The toxicity of DEHP may be summarized by the statement that the compound is relatively non-lethal by any route. At very high dosages, some differences in toxicity have been reported but these may have been due to strain differences or batch to batch variability of the plasticizer that was being tested. Only one aspect of toxicity is present in most studies and that is the finding that DEHP is a moderate irritant.

It is this latter property which causes peritoneal irritation and adhesion formation on ip injection, causes dye extravasation when injected subcutaneously before a systemic dose of dye, and weight loss when fed at high levels. However, these effects do not provide sufficient

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evidence to warrant a restriction on the use of DEHP in those instances where films of plasticized materials are exposed to substances which do not cause a significant degree of extraction.

H. Butyl Glycolylbutyl Phthalate - Oral Toxicity

Lefaux (1968) reports that the material has been given to rabbits at an oral dose of 2.10 gms/kg with no ill effects. Similarly, doses of 3.2 - 4.7 grams/kg were given to rats with no ill effects. It is also reported by Lefaux that Hazelton laboratories studied the effect of BGBP fed to rats in their diet. At the levels of 0.02, 0.2, and 2%, the only ill effects observed were at the 2% level after one year. This level caused a retardation of growth. Histological examination of all animals disclosed no abnormalities.

I. Butyl Glycolylbutyl Phthalate - Intraperitoneal and Intravenous Toxicity

It has been reported by Trimble et al (1966) that a lipid isolated from the fat of hibernating animals could prolong the survival of animals exposed to cold. This lipid material was later found to be a contaminant of the isolation procedure, and was identified as BGBP. Doses of 500, 1000, and 1500 mgs given intraperitoneally as a sterile emulsion to adult guinea pgis were not immediately fatal. Dogs were also treated with intravenously this emulsion and no immediate deaths were reported. No dose was mentioned; late mortalities due to cold exposure prevented any evaluation of the lethality of the plasticizer.

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Guess and Haberman (1968) report that BGBP given to mice intraperitoneally in an undiluted volume of 0.5 ml did not cause stretching or other signs of abdominal irritation nor did it cause any deaths. This dose is equivalent to 27.4 grams/kilo. Therefore, the LD_{50} in this species must be greater than this value.

J. Butyl Glycolylbutyl Phthalate - Miscellaneous Toxicity

Guess and Haberman (1968) in their report on plastics additives found no irritant effect of saturated solutions of BGBP in the rabbit based on the dye extravasation method. This material did not affect chick embryo cells or mouse fibroblasts when applied undiluted or as a saturated saline solution. However, the growth of amnion cell and nasopharyngeal cancer cells was enhanced. Saturated saline solutions of BGBP were not hemolytic to red cell suspensions.

K. Summary - Use of Plastics in Medical Devices

These plasticizing agents have been found without lethal effect under the conditions tested, and they are approved for use in food packaging materials (Food Additives Amendment, 1968). Autian, Berg, and Guess (1966) have stated that "great emphasis has been placed on approved FDA additives and a number of manufacturers or fabricators of medical plastic items have felt that FDA approval signifies 'safe for medical use'". These authors state that such reliance is usually based on oral toxicity studies and is not adequate for the testing of a medical device.

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Medical plastics testing is specified by the USP (1965), which requires that eluates of the material be tested by iv and ip administration. It is also specified for some plastics that the material must be implanted into tissue of the rabbit to determine the formulation's tissue compatability. However, these tests of medical plastics do not always simulate the conditions of use. Therefore, such tests, although valuable, do not necessarily reflect the safety of the material when humans become the test subjects. Further, USP standards are not laws, and presently, medical device legislation (S. 2107) introduced by Senator Nelson is not yet approved by the Congress. Thus no legislation exists to regulate the materials used in plastic medical devices.

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III. METHODS

A. Isolated, Perfused Rat Liver

The procedure for the isolated perfused rat liver was essentially that of Miller et al (1951). The perfusion apparatus was purchased from Blessig Glass Specialties Company, Rochester, New York. Rat blood was obtained from retired, male breeder rats (400 to 600 + gms) that were obtained from Carworth Farms, New City, New York as needed. The blood was drawn from etherized animals by cannulation of the abdominal aorta with PE-190 catheter tubing, or was drawn from the same site using heparinized syringes. Five thousand units of heparin was added to the total of 70 ml of blood drawn. To make the solution used for the liver perfusion, this blood was mixed with one half volume of a 4% bovine serum albumin solution (Nutritional Biochemicals Corp., Cohn fraction V powder) dissolved in Krebs-Ringer Bicarbonate buffer (Umbreit, 1957). The final mixture, 105 ml, was filtered through one layer of cheese cloth prior to use.

The perfusion apparatus was assembled from glassware which had been washed in chromic acid cleaning solution. Tubing was of polyvinyl chloride, silicone or gum rubber. The former material was obtained from The Norton Company, U.S. Stoneware Division and was of type S-22-1 or S-50-HL. Silastic tubing (TM) was obtained from the Dow Corning Company, and gum rubber was from Fisher Scientific. All tubing was of Surgical grade.

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The apparatus was filled with 100 or 250 ml of saline (50 U Heparin per ml) and was allowed to circulate for thirty minutes. It was found that the total amount of saline which could not be recovered from the system was independent of the amount added. In most cases, approximately 20 ml of saline were left behind. Later experiments, therefore, utilized the larger saline volume in order to wash the system completely and to leave the least residual contamination. The perfusion system contained two in-line filter assemblies. The first, in the "arterial" line filtered all blood being pumped. The second filter was placed immediately before the liver and only filtered blood about to enter the liver.

The pump, a variable speed Sigma finger pump, was placed below the level of the bottom reservior. Blood was never pumped at a negative pressure. Oxygenation of the system was via a falling film, multi-lobed glass lung which was gassed with humidified 95% 0_2 and 5% CO_2. The entire system was enclosed and maintained at 37°C.

Perfusion of the liver was at constant pressure, 18 cm H_2O, and the liver was allowed to adjust its own flow at this pressure. Perfusate in excess of the liver's needs was bypassed by an overflow system, and was allowed to return to the main reservoir. Blood and bile flow were measured and these were taken as functional indicators of the liver's condition. Perfusate pH was maintained by the periodic addition of $NaHCO_3$.

The liver donor, a male Carworth Farms rat weighing between 250 - 325 gms, was anaesthetized with pentobarbital solution (45mg/kg).

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The liver was exposed by a midline abdominal incision, and the bile duct was cannulated with PE-50 tubing. The animal was heparinized with 500 units of heparin which was allowed to circulate for 3 minutes prior to cannulation of the portal vein. This was done with a number 15 gauge teflon needle, and the liver was perfused with iced saline containing 10 units of Heparin per ml (saline gassed with 100% 0_2). Major vessels were tied off and a short PE-300 cannula was placed in the superior vena cava. The liver was freed from its adhesions and transferred to the perfusion chamber. This procedure usually involved from five to seven minutes from the time that the portal vein was tied off for cannulation to the time of perfusion.

The liver was connected to the perfusion system and blood was allowed to re-enter the liver. Thirty minutes were allowed as the equilibration time required by the liver for recovery to near normal values of blood and bile flow. Measurements were made from this time as the time zero value, or from time zero, measured from the onset of perfusion.

B. Perfusion Fluid Extraction and Column Chromatography

Samples of the perfusion fluid were withdrawn from the perfusion apparatus at timed intervals. Alternatively, the entire perfusate was collected at the end of the perfusion, usually four and a half hours. The perfusate was centrifuged at 600 x g for 30 minutes, and the plasma was collected. An equal volume of ice-cold 10% perchloric acid was added, and the precipitate removed by centrifugation.

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The resultant 5% PCA extract was neutralized at 4°C with ice-cold potassium hydroxide (sufficiently concentrated to make the total volume added quite small.) The supernatant was isolated by filtration through Whatmann No. 4 or by centrifugation. The resulting neutralized solution was then further extracted or was fractionated by column chromatography.

Further extraction of the neutralized supernatant was done with diethyl ether. The neutralized solution was reacidified with HCl, and extracted several times with ether. The ether extracts were pooled, and chilled to -20°C. This was done to remove any trace amounts of water and its attendant water-soluble materials. Subsequent to this, the ether was evaporated to a small volume, and an aliquot of distilled water (10 ml) was added followed by total removal of the ether by gentle heating. By this method, all acidic ether-soluble materials are transferred into water. To this solution, an aliquot of radioactive ^{14}C AMP and ^{14}C ADP were added. These materials would serve as a marker throughout further chromatographic separation.

Separation of the aqueous acidic components was accomplished through the use of anion exchange chromatrography. Columns of Biorad AG 1 x 8 anion exchange resin in the formate form were prepared. These columns were 0.7 by 10 cm in size and the sample was applied either as the neutralized plasma extract or as the aqueous solution from the ether extract. It was found that this latter step improved separation as the neutralization process does not remove all the dissolved

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potassium perchlorate.

The sample and its associated markers were applied to the column and the non-adherent materials were washed off the column with forty ml of water. Elution was via a non-linear gradient of 0-2N ammonium formate (pH 5.0). Ultra violet absorbance was followed at 260 nm with a Gilford recording spectrophotometer, in a continuous flow cell. Flow through the column was approximately 30 ml/hr and fractions were collected at 30 minute intervals. ^{14}C was counted in a Packard Tri-carb liquid scintillation spectrometer.

Subsequent to elution from the column, the unknown material was isolated from the eluting buffer by ether extraction of the acidified solution. After removal of water by freezing, the ether was removed and water added. This was lyophillized to remove the formic acid. The purified, unknown material appeared as a white powder.

C. Synthesis of Glycolyl Phthalate

Phthalic acid (0.1 mole) and glycolic acid (0.1 mole) were dissolved in 200 ml of anhydrous dioxane. To this was added 0.1 mole of dry pyridine (7.8 ml) and the resultant solution was refluxed for four hours.

The solution was allowed to cool and the dioxane and pyridine were removed by gentle heating (60°C oven) at reduced pressure. It should be noted that glycolyl phthalate is thermally unstable and will spontaneously break down to glycolic acid and phthalic acid (Monsanto

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Chemical Company personal communication). Thus care should be taken that excessive heat be avoided.

After removal of solvent, the residue was dissolved in 1% $NaHCO_3$. This was washed with ether to remove phthalic anhydride. The solution was then acidified with HCl and extracted with diethyl ether. Glycolyl phthalate and phthalic acid, both present in the ether phase, were fractionated by their differential binding to an anion exchange resin.

An aqueous aliquot of glycolyl phthalate and phthalic acid solution was applied to an anion exchange column of suitable capacity. This was eluted with stepwise gradient of ammonium formate, and the UV absorbance monitored. That material eluting between 0.8 and 1.2 N ammonium formate was glycolyl phthalate. Phthalic acid eluted above 1.4 N and was discarded. The purity was confirmed by the serial dilution technique when compared to the smallest amount of phthalic acid which could be detected by thin layer chromatography (vide infra).

D. Thin Layer Chromatography

The plate of silica gel containing a fluorescent phosphor (254 nm excitation) was 250 microns thick on glass (Brinkman Instrument Company), and was activated at 125°C for 30 minutes just prior to use.

The plates were spotted and the spots quickly air dried. Development was in solvent-saturated tanks at room temperature using one of the following solvents at the indicated volume ratios.

I Butonal-acetic acid - H_2O (70 - 20 - 10)

II N-proponal-ammonium hydroxide (70 - 30)

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III Methylene Chloride (100%)

IV Iso-octane - ethylacetate (90 - 10)

The Rf values in these solvents were:

	I	II	III	IV
Phthalic acid	.547	.146	--	--
GP	.421	.207	--	--
DEHP	--	--	.463	.368
BGBP	--	--	.276	.148

E. Sleeping Time Determinations

1. Procedure. Male Carworth Farms rats or mice were received in the laboratory in the weight range of 175 - 200 grams (or 15 - 20 gms respectively). They were allowed tap water and Rockland rat-mouse lab chow ad lib and were resident in the laboratory for at least five days before being used in the determinations. They were usually in the range of 200 - 225 gms (20 - 25 gms for mice) at the time of the experiment.

Animals were pretreated (ip or iv) with the plasticizer suspended in a vehicle of 3% acacia in saline. The dose was designed as 1 ml/100 gms of body weight and contained 50 mg DEHP/ml of solution (500 mg/kg). Doses were obtained by adjusting the volume of solution given. These pretreatments were given 30 minutes prior to hexobarbital or zoxazolamine administration.

2. Hexobarbital. Hexobarbital in the acid form, was obtained from K and K Labs and was prepared fresh on the day of use. 732 mgs

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of the material was weighed out and 10 ml of 0.31 N NaOH in normal saline was added for each 732 mgs weighed out. This was stirred with occasional heating under warm tap water until the solution was clear. The final solution was always filtered prior to use. All animals were injected intraperitoneally at the level of 60 mg/kg in mice or 250 mg/kg in rats. Sleeping time was measured as the time from injection to the time that the animal righted itself three times consecutively.

3. Zoxazolamine. Zoxazolamine paralysis time was determined with the animals having been pretreated 30 minutes before the administration of the paralytic drug. Zoxazolamine was given at the level of 75 mg/kg and was prepared as follows: 200 mgs zoxazolamine were weighed out and 2.3 ml of N/1 HCl and 7.7 ml of saline were added. This solution was stirred till completely dissolved and animals were injected a dose of 0.38 ml/100 gms.

F. Hexobarbital Assay

Hexobarbital was assayed by the method of Cooper and Brodie (1955) as modified in this laboratory. Samples of blood or perfusate and appropriate hexobarbital standards dissolved in blood were placed in 50 ml round bottom flasks to which had been added 1.5 vols of 0.5 M citric acid saturated with NaCl. 20 ml of 1.5% isoamyl alcohol in heptane was added by syringe or graduate. The tubes were capped, shaken rapidly (200 osc/min) for 1 hour and spun at 600 xg (4°C) for 30 minutes. 15 ml of the upper organic phase was transferred to clean tubes and a

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volume of pH 11 phosphate buffer was added. The tubes were capped and again shaken for 1 hour; subsequently, they were centrifuged at room temperature for 30 minutes at 600 xg. Optical densities were read at 245 nm with pH 11 buffer as the blank.

Amounts of hexobarbital were determined on a mg/ml basis from the standard curve prepared in blood and obtained with each assay.

G. Zoxazolamine Assay

Zoxazolamine was determined by the method of Burns (1958). Blood samples were drawn in heparinized glass syringes from the abdominal vena cava immediately after the animal had righted itself three times. Two ml of blood and 3 ml of 0.5 N NaOH and 30 ml of ethylene dichloride were shaken together for one hour.

The tubes were spun and the upper phase and clot were aspirated. Twenty ml of the lower phase was transferred to a clean flask and 3 ml of N/1 HCl was added.

This mixture was shaken for 30 minutes, centrifuged at room temperature for 5 minutes and the optical density at 278 nm was read relative to N/1 HCl. The zoxazolamine content per ml of blood was determined from a standard curve of zoxazolamine dissolved in blood and determined with each experiment.

H. Quantitation of Di-2-ethylhexyl Phthalate

Samples of tissue or body fluids were weighed or their volumes

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noted. The samples were then lyophillized. Approximately 20 or more volumes of chloroform-methanol (2:1 v/v) were added, and the tissues were minced with scissors or homogenized in a blender. Fluid samples were lyophillized in polyethylene bottles and the extraction was performed in the same bottle.

The mixture was allowed to stand for 24 - 72 hours and occasionally during this time, the containers were shaken or swirled. To insure complete extraction, the tissues samples may be removed and re-extracted in fresh solvent. This was not usually necessary with homogenized tissues or lyophillized body fluids.

The extracts were filtered through Whatman No. 4 filter paper and the extracting vessel, residual bits of tissue and filter paper were rinsed with the extracting solvent. To this combined extract was added an equal volume of saline. The resulting aqueous methanol concentration was 25%, a concentration at which DEHP is not readily soluble. It should be noted that DEHP is slightly soluble in 62% methanol such as results from the standard Folch procedure (Folch et al, 1957). The larger volume of saline was used to lower the methanol concentration and thus to avoid any losses of the DEHP.

The two phase system that had formed was allowed to stand for several hours with occasional swirling or if the volumes were small, the contents were centrifuged. Following phase separation, the upper aqueous phase was aspirated and discarded, and the lower chloroform phase collected. This was done by filtering the lower phase through Whatman

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phase separating paper (1PS, Reg T.M.) and the flasks and filter were rinsed with chloroform. The chloroform, following bulk treatment with 1 - 2 gms silicic acid (Unisil, Clarkson Chemical Company), was collected by filtering through Whatman No. 4 paper. Again flasks and filter were rinsed with chloroform.

The chloroform was dried under a stream of nitrogen and gentle heating (60°C water bath) until it could be transferred quantitatively to a 15 ml centrifuge tube. The chloroform was taken to complete dryness in this tube and approximately 10 ml of methanol added, depending on the total amount of lipid remaining in the tube. The tube was centrifuged at 600 - 1000 xg for one to two hours at 4°C. The methanol phase was decanted, and the methanol was removed under nitrogen with gentle heating and the sample was ready for gas chromatographic analysis. The conditions for GC analysis were as follows:

Injector	250°C
Column	225°C
Detector	270°C
Column	6' x 1/4" 3% SE-30
	on Gas-Chrom Q (80 - 100 mesh)
Carrier gas	Nitrogen, 40 - 60 ml/min at 50 lbs.
Detector	Flame Ionization
Hydrogen flow	40 ml/min at 20 lbs.
Air Flow	400 ml/min at 20 lbs.

The sample was dissolved in hexane, at a volume which

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depended on the amount of lipid present. The amount of DEHP present was quantitated by comparison with absolute standards and/or was compared to standards carried through the entire extraction process. Internal standard recoveries ranged from 60 - 98% depending on the volumes involved, the rigorousness of rinsing, etc.

I. Methylation of Organic Acids

Phthalic acid or glycolyl phthalate in dried diethyl ether was methylated by the procedure of DeBoer as detailed by the Aldrich Chemical Company (1970) using Diazald as the source of the methylating agent, diazomethane. Ethereal solutions were prepared fresh,as needed,according to the following procedure.

Twenty-five ml of 95% ethanol was added to a solution of potassium hydroxide in distilled water (5 grams KOH in 8 ml of H_2O). This was placed in a round bottom distilling flask to which had been added several glass beads. 21.5 grams of Diazald (0.1 mole) dissolved in approximately 200 ml ether, was added slowly to the ethanolic base via a dropping funnel. Using gentle heating with a 60°C water bath, the ethereal diazomethane was distilled through a short, water-cooled condenser into a flask chilled in ice. After completion of the Diazald addition, sufficient ether was added to the reaction flask to insure that all the diazomethane had distilled off.

The ethereal diazomethane solution (approximately 3 grams in 250 ml ether) was used promptly after being made.

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J. Quantitation of Phthalic Acid

Phthalic acid was extracted from aqueous solution as previously described. Following removal of water from the ether, the organic acids were treated with an ethereal solution of diazomethane and converted to their methyl esters. The excess, unreacted diazomethane was allowed to evaporate in a fume hood. The ether phase was washed with pH 11 phosphate buffer (Cooper and Brodie, 1955) to remove unreacted organic acids. In order to insure that all phthalic acid had been methylated, suitable standards of phthalic acid in ether were also methylated. Recovery of these (usually 100 mgs) as dimethyl phthalate was 98%. Suitable standards of phthalic acid were also extracted to insure complete recovery of the original aqueous solution of phthalic acid.

The ether was evaporated; methyl esters were then dissolved in acetone and assayed by gas chromatography. The GC was used isothermally under the following conditions:

Injector	250°C
Column	125°C
Detector	270°C
Column	6' x 1/4" 3% SE-30 on Gas-Chrom Q (80 - 100 mesh)
Carrier gas	Nitrogen, 30 ml/min at 50 lbs.
Detector	Flame ionization
Hydrogen flow	20 ml/min at 20 lbs.
Air flow	200 ml/min at 20 lbs.

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Urine samples from one patient were treated according to the method of Shaffer et al (1945). Seventy-five ml aliquots of the patient's total daily urinary output was placed in a flask and 5 ml of 10% sodium hydroxide was added. The mixture was taken to dryness by heating. The sample was transferred to a 100 ml Kjeldahl flask with the aid of 25 ml distilled water and 25 ml concentrated nitric acid. To this was added several glass beads and 0.2 grams of copper nitrate. The mixture was refluxed for four hours after which it was cooled. 50 ml of distilled water was added and the solution was saturated with ammonium sulfate (ca. 55 gms). It was washed twice with 25 ml reagent grade chloroform, and the washes were discarded. Four extractions of 50 ml each were made with diethyl ether. These were pooled and washed with 50 ml of saturated sodium chloride solution. The ether solution was further washed with 0.02 N HCl. The resultant washed ether solution was treated with silicic acid and chilled to -20°C. Subsequent to this, the dried ether phase was treated with diazomethane and the resultant dimethyl phthalate determined as previously described.

K. Hypothalamic Electrode Implantation

With chronically implanted brain electrodes, it is possible to train a rat to perform a task. Thus, male hooded rats of the Long-Evans strain (300 gms) were anesthetized with pentobarbital (45 mg/kg). The skull was immobilized in a stereotaxic mount, and the scalp was shaved and washed with an antiseptic solution. A midline incision was made

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above the bregma and the coordinates of Olds (1963) were employed: the electrode was implanted 4.5 mm posterior of the bregma, 1.5 mm to the right of the midline, and 8.5 mm below the skull. Thus the tip of the electrode could be placed directly into the hypothalamus. The bipolar electrodes were cemented into place with Duralay dental cement.

During the one week following surgery, vigorous antibiotic therapy was employed while the animals recovered. After this time, they were placed in a Skinner box and were trained to depress a lever which gave them a pleasurable electric current. This hypothalamic current was sufficient to promote the learned lever pressing task, and animals who did not learn the task were discarded. Response rates in the trained animals were adjusted to 20 - 100 responses per minute by altering the level of current that they received. The current levels were from 20 - 60 uA at 28 volts A.C.

L. Activity Wheel Measurements

Male albino rats in the 300 gm range were allowed to live continuously in activity wheel cages and were given food and water ad lib. Their activity (revolutions per 24 hours) and body weight were measured at 12 noon each day.

M. Screen Filtration Pressure

Samples of canine blood were stored in polyvinyl chloride blood bags (Fenwal, Morton Grove, Ill), silicon-lexan copolymer bags

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(Hemosil 103, Medical Engineering Corp, Racine, Wisconsin) or specially prepared PVC bags having an inner surface of heparin and graphite (Courtesy Dr. V. Gott, E. Klopp, and M. Petracek, Johns Hopkins Hospital). At suitable times samples were withdrawn and the screen filtration pressure evaluated by the procedures of R.L. Swank (1961). This method measures the pressure required to cause blood to flow through a 20 micron screen at constant flow. At the same time, samples of blood were evaluated for their DEHP content. This was done by methods previously described.

N. Carbon Clearance Measurements

Rats in the 200 gram range were pretreated with DEHP or 3% acacia in saline (iv). The DEHP was given at a dose of 125 or 500 mg/kg (DEHP concentration of 50 mg/ml.) The emulsion was prepared by sonication. Injections were given intravenously via the dorsal vein of the penis while the animals were under light ether anesthesis.

A carbon suspension (C11/1431 a) from Gunther Wagner, Hanover, Germany as prepared by the methods of Biozzi et al (1953) was purchased from John Henschel and Co, Farmingdale, New York. The rats were anesthetized with pentobarbital at a dose of 45 mg/kg (ip), and the carbon dose was given intravenously in the penis vein at a dose of 40 mg/kg. Thirty microliter blood samples were taken in heparinized micro pippettes (Drummond micro capillary tubes) from the cut end of the tail. Samples were taken at two minute intervals (2, 4, 6, 8, 10 or 1, 3, 5,

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7, 9 minutes). The blood was lysed in 2 ml of 0.1% $NaCO_3$, and the optical density of this solution at 750 nm was taken as a measure of the carbon remaining in the blood.

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IV. EXPERIMENTAL RESULTS

A. Appearance of an Unknown Compound in the Isolated Perfused Liver System

Experiments were performed utilizing the isolated perfused liver of the rat. Samples of the liver were removed and a perchloric acid extract was made. This was neutralized with KOH and applied to an anion exchange column. The column was eluted with a non-linear gradient of ammonium formate. It can be seen in figure one, that in addition to the expected nucleotides, UMP, AMP, ADP, and ATP, an identified peak was seen between the peaks associated with ADP and ATP. The unidentified material absorbed ultra-violet light at 260 nm and was further defined in its elution relative to ADP. A comparable peak could not be observed when samples of liver were assayed immediately after removal from the animal without subsequent perfusion.

When sequentially biopsied liver samples were assayed chromatographically, it was found that the hepatic concentration of the material increased with time of perfusion. The time course of accumulation in the perfused liver is shown in figure two.

In addition, samples of perfusate from a 4.5 hour liver perfusion were found to contain an unidentified material similar to that observed in liver. The material from both sources had a similar UV spectrum, and an identical volume of elution relative to ADP. However, as in the liver, this substance was not present in freshly prepared perfusate. From

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Figure 1. Perfusion experiments were done as described in methods. In this experiment, samples of liver were biopsied, and a 25% homogenate in saline was prepared. This was made to 5% PCA concentration and centrifuged. The acid soluble extract was neutralized with KOH, and was applied to a Dowex-1 anion exchange resin. Elution of the column was as described in methods. Labeled nucleotides were added to the neutral extract to serve as markers. In this figure, only optical density is shown. Volume is represented by tube numbers.

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APPEARANCE OF AN UNIDENTIFIED MATERIAL IN THE ISOLATED PERFUSED RAT LIVER

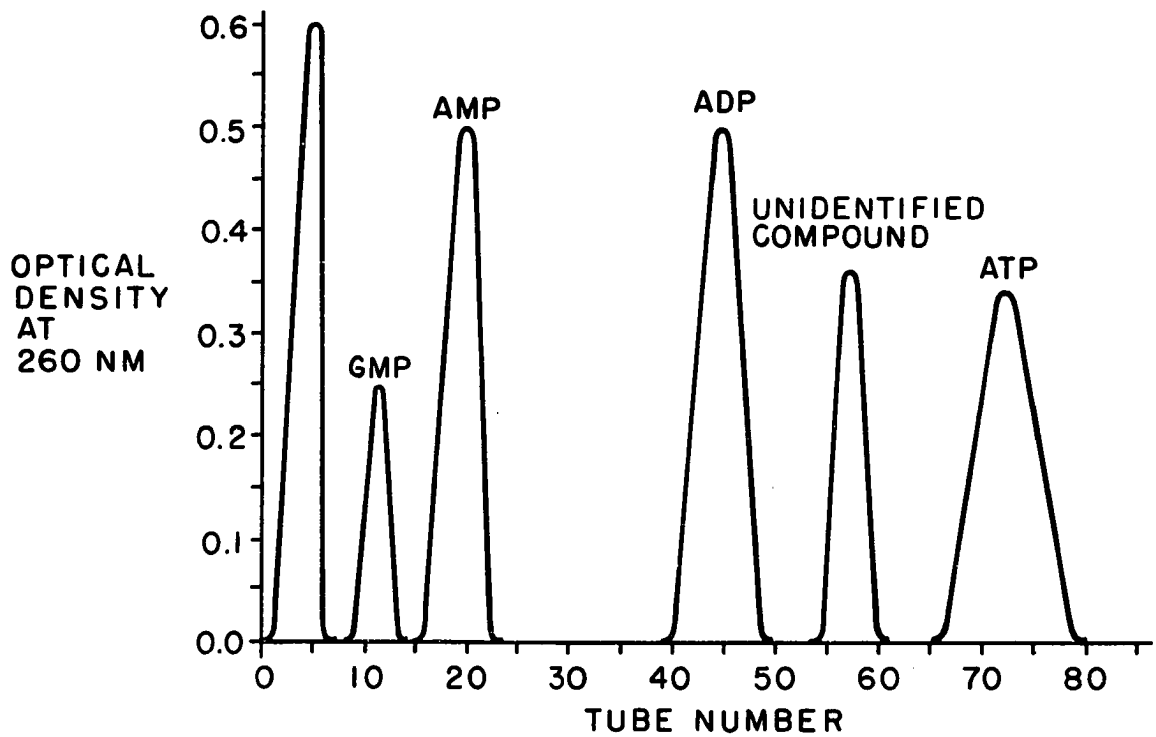

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Figure 2. A single perfusion experiment was done as previously described. Biopsied samples were removed at timed intervals, and extracts prepared and analyzed as previously described. The amount of the unknown compound present in the liver is represented by the peak optical density at 260 nm. The same amount of initial liver weight was used for each determination.

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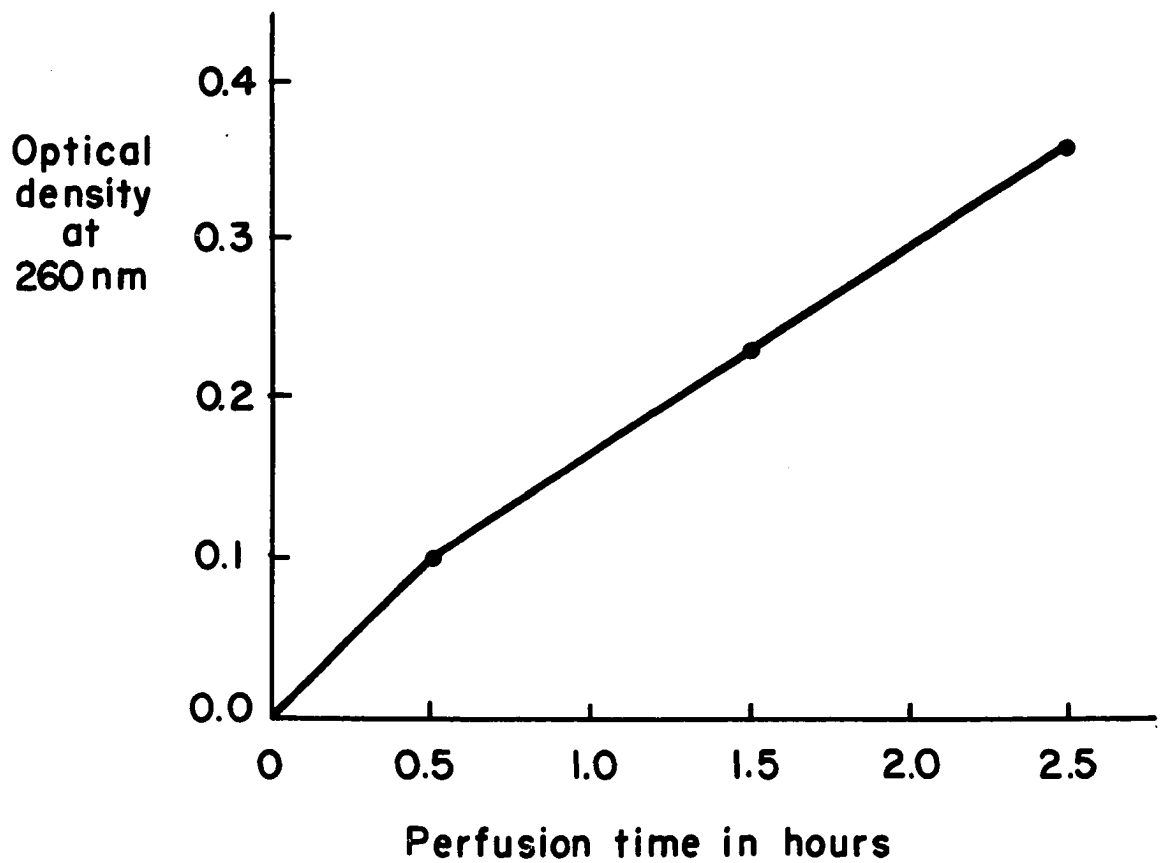
Time course of appearance of the unidentified material in the isolated perfused liver.



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this information, therefore, it was concluded that the appearance of the unidentified material was somehow dependent on the perfusion system.

An experiment was performed to determine the origin of the unknown material. Perfusate was circulated in the apparatus for four and a half hours in the absence of a liver, and the acid soluble extract of this was chromatographed and compared to a similarly treated extract of perfusate from the 4.5 hour perfusion of an intact liver. The result of this experiment is shown in figure three.

The dotted lines of this figure indicate the accumulation of substances in the perfusate in the absence of a liver, while the solid line indicates those compounds which appear in the presence of a liver. The lines labeled AMP and ADP are representative of amounts of ^{14}C labeled nucleotides which were added to the extract to serve as a chromatographic marker during fractionation.

It can be seen from the figure that the peak labeled I is common to both experimental conditions. The compound represented by this peak was identified as uric acid and probably results from the action of plasma xanthine oxidase on circulating purines. Peaks II and III appear only when an intact liver is in the system and Peak III appeared to be the same as the unidentified material found in samples of perfused liver. Thus, the appearance of the unidentified material depends on the presence of an intact liver and the perfusion of that liver within the apparatus.

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Figure 3. Circulation of perfusion fluid in the presence (————) and the absence (--------) of a liver. The prefusion fluid, a mixture of 70 ml of whole rat blood containing 70 units of heparin per milliliter and 35 ml of Krebs-Ringer-bicarbonate buffer containing 4 percent BSA and 80 mg of glucose per 100 ml, was circulated in the liver perfusion apparatus of Miller et al (1951). This system was used to perfuse an isolated rat liver for 4.5 hours or the perfusion fluid was allowed to circulate in the absence of a liver for the same length of time. At the end of the experiment, the total plasma was isolated by centrifugation and acidified with perchloric acid. After removal of the precipitate, the acid-soluble supernatant was neutralized with KOH and centrifuged, and the supernatant recovered. An amount of (^{14}C)adenosine diphosphate and (^{14}C)adenosine monophosphate was added to the neutralized extract to act as a marker during further chromatographic fractionation. The total extract was applied to a 0.7 by 10 cm column of Dowex-1 (formate form) anion exchange resin. Elution of the column was with a nonlinear gradient of ammonium formate (0 to 2N, pH 5.5), and the absorbance at 260 nm was monitored continuously in a Gilford spectrophotometer. Portions of each fraction were counted in a Packard TriCarb liquid scintillation counter. In order to simplify this figure, only the peak radioactive fractions are displayed.

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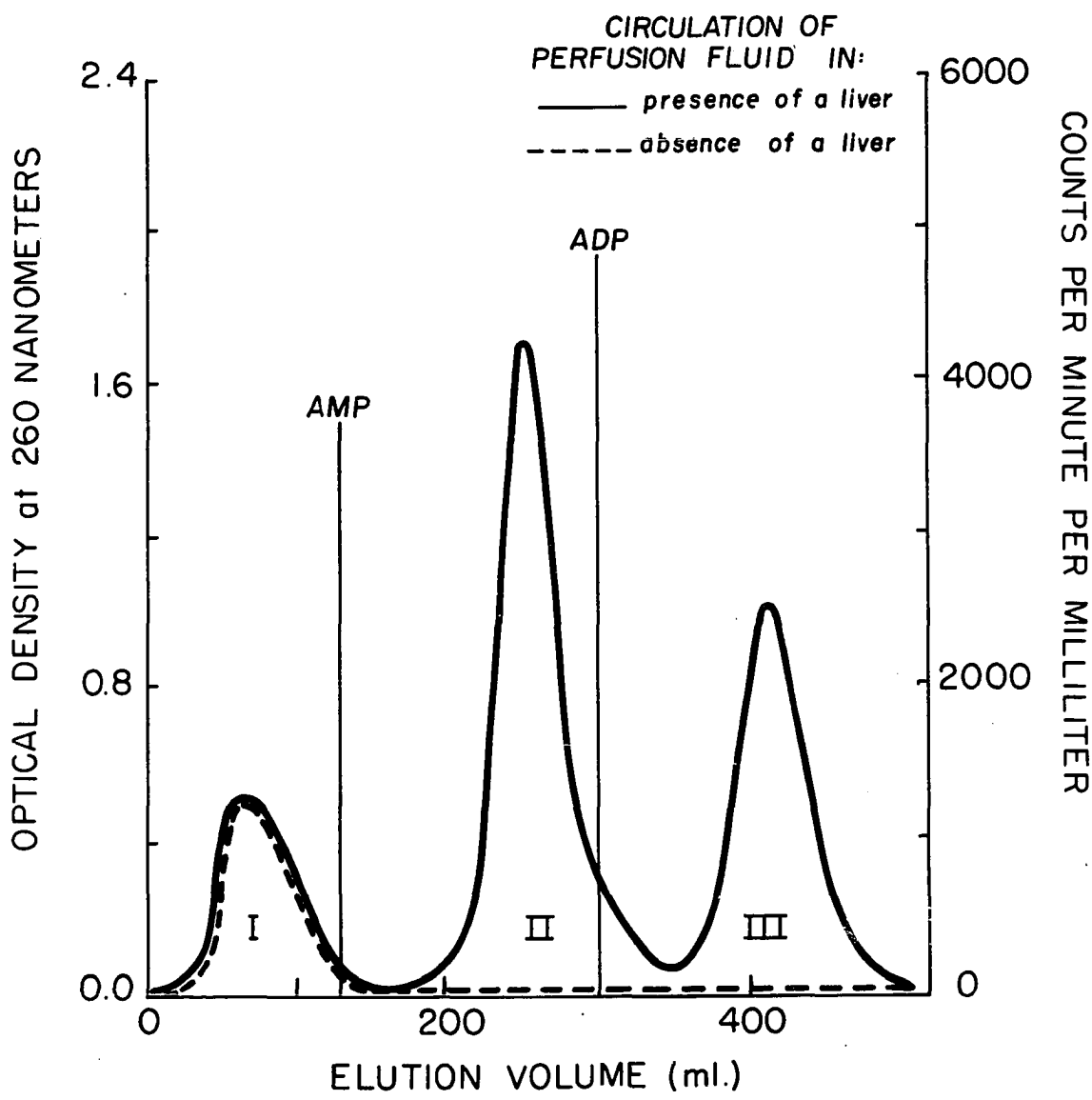
Appearance of the unidentified material in perfusion plasma



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B. Isolation and Identification of the Unidentified Material

The ultra-violet absorption spectrum of the unknown compound was determined in ammonium formate buffer. It was found that while the material had a significant absorbance at 260 nm, this value represented a minimum of absorbance, and a peak absorption was seen at 280 nm. No spectral information could be obtained below 250 nm because of the interfering end absorption due to the buffer. However, from the available data, the 280/260 nm ratio could be calculated. Comparison of the absorbance spectrum and its 280/260 ratio with similar data for other compounds in the literature did not permit the rapid identification of the unknown material as a commonly occuring bio-molecule. Therefore, attempts were made to isolate the compound in pure form for further study.

Various desalting techniques were attempted and it was found that the unknown compound was extractable from the eluting formate buffer if the solution was acidified, and extracted with diethyl ether. The ether phase then contained formic acid and the unknown material. This extraction characteristic was consistent with the compound being an organic acid.

When the ether extract was dried and lyophillized to remove formic acid, the UV spectrum of the material in distilled water could be measured. This spectrum in acidic and basic solution is shown in figure four. It can be seen that there is a second peak at 230 nm which is altered by changes in pH, and could represent the presence of an ionizable group near the UV absorbing chromaphore. This theory was strengthened by

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Figure 4. The unidentified material was purified by anion exchange chromatography and was freed of the eluting buffer by acidification and diethyl ether extraction. The ether solution was taken to dryness, and freed of residual formic acid by lyophilization. A quantity of water was added such that the final concentration of the unidentified material was approximately 25 µg/ml and absorbance was determined in a dual beam Spectronic 505. The pH was adjusted by the addition of small volumes of concentrated HCl or NaOH.

Gravimetric analysis of the purified material indicated that 0.18 mgs when dissolved in 1 ml of distilled water had an optical density of 1.0 at 278 nm. This constant (0.18 mg/O.D. unit 278 nm) was used to determine the amount of material present in solutions of known optical density.

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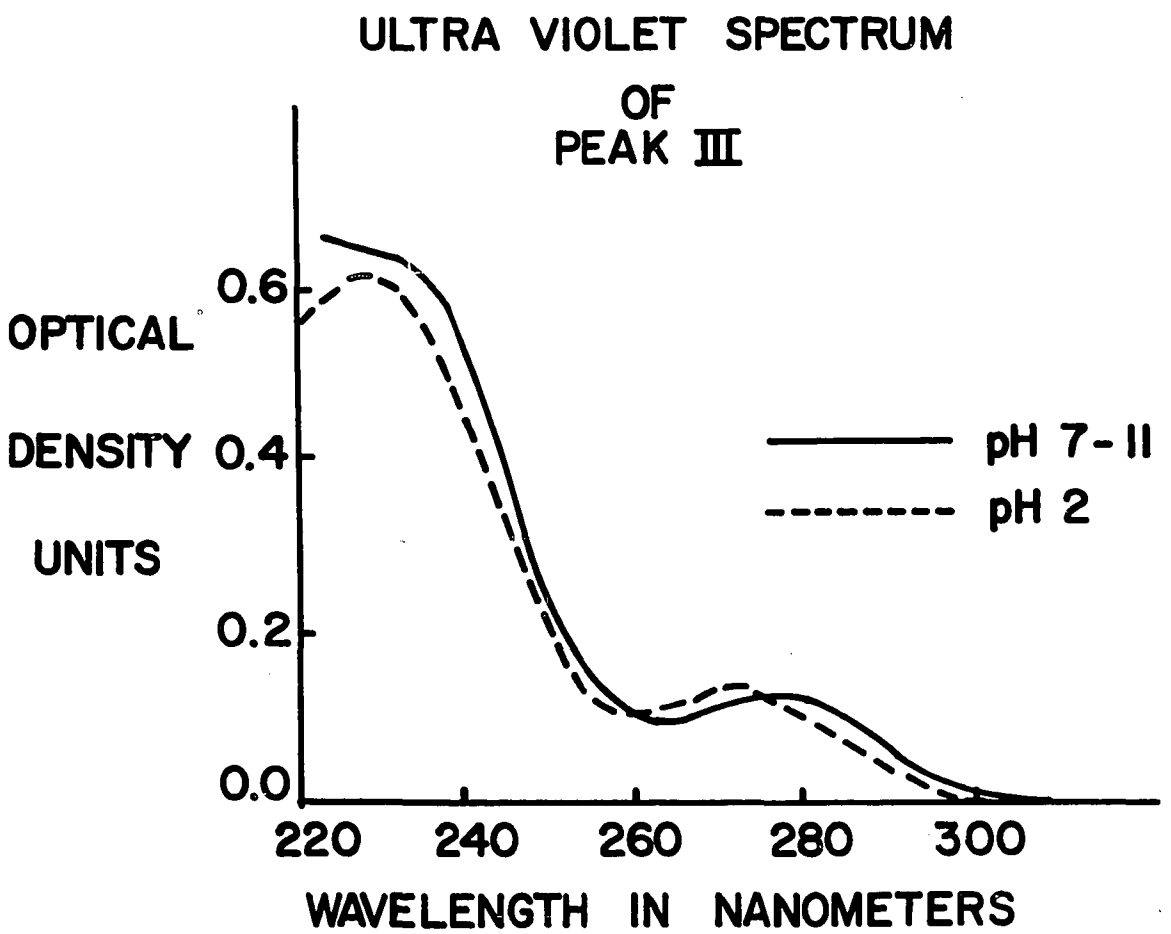

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the observation that extraction of the compound did not occur if the pH of the aqueous phase was greater than pH 5. Such a property was considered consistent with the presence of a negatively charged group as might be expected in a carboxylic acid, the presence of which could explain the binding to the anion exchange resin. Chemical analysis for the presence of phosphate was negative as were tests for nitrogen.

The peak observed at 280 nm in neutral solution could represent the absorption of a carbonyl group, and thus the hypothesis that the material was an organic carboxylic acid was quite likely. In order that more sophisticated analytical tests could be performed, it was decided to make a derivative of the compound with an agent which is relatively specific for carboxylic acids, the methylating agent, diazomethane. When ether extracts of the unknown compound were treated with this reagent it was found that the compound was no longer extractable from ether into basic solutions. Further, when the ether extract containing the diazomethane treated compound was taken to dryness, the material was no longer water soluble at any pH. Thus, the molecule had been altered by treatment with diazomethane and it, was likely to have been methyl esterified.

A large quantity of the material, thus prepared, was divided and submitted for nuclear magnetic resonance spectroscopy and mass spectral analysis. The NMR spectrum obtained is shown in figure five. By this technique, the number and type of protons in a molecule can be measured. The shift of peaks from a standard reference compound,

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Figure 5. The unidentified compound was methylated with diazomethane, and the reaction product was dissolved in carbon tetrachloride. Concentrated solutions were prepared and submitted for NMR analysis in a Varian 60 MHz. spectrometer. Internal trimethyl silane standards were added prior to analysis, and the concentration of the standard was adjusted to the range of the unknown material. The concentration of the unidentified material in this solution is not known, but the instrumental sensitivity was adjusted so that readings could be obtained.

Integration of the peaks was by triangulation, and the nearest whole number ratio of protons was used to determine proton number.

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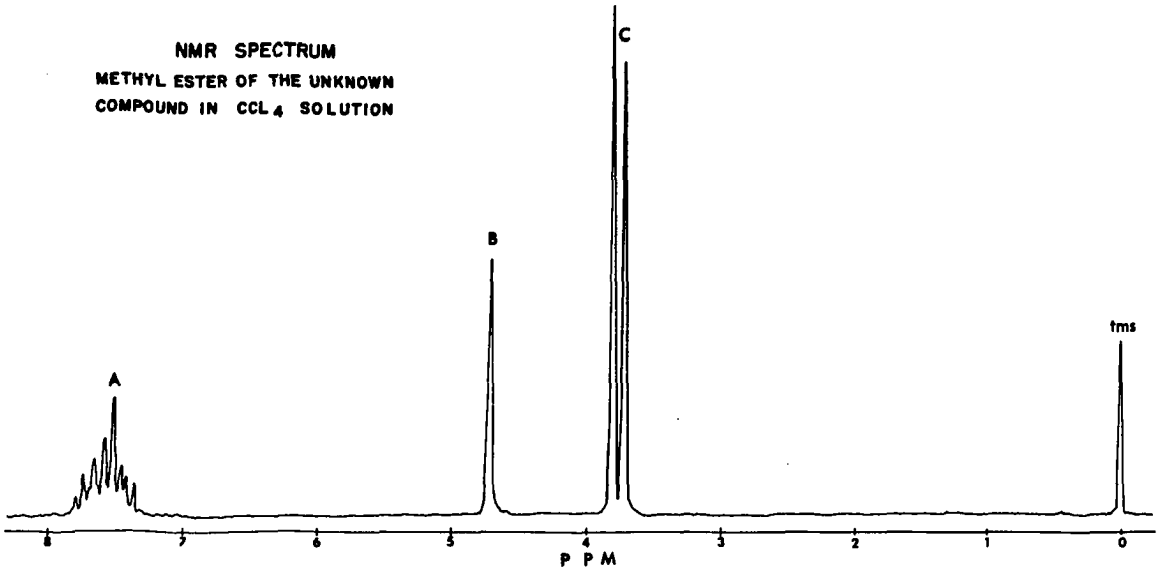

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trimethyl silane (TMS), serves to identify the proton type, and the integrated area of each peak yields a quantitative measure of the relative numbers of each type of proton. As can be seen in the figure, the methyl ester of the unknown compound contains three types of protons. The peaks labeled A correspond to four aromatic protons arranged ortho to one another. Peak B represents two protons, probably of the methylene ($-CH_2-$) type. However, their position relative to TMS is such that there may be some strongly electron withdrawing group adjacent to them, an effect which might be expected if the methylene protons were near to carbonyl groupings. The third proton type is due to methyl ester protons as indicated by the double peaks at 3.8 ppm. The area under the double peaks indicates that six protons of this type are present, and suggests the presence of two methyl ester groups. The peaks are not coincident, a result which indicates that the molecular environment of the two esters are slightly different from one another.

The mass spectral fragmentation pattern of the methyl ester is shown in table two. Possible structures for these fragments are also shown in this figure. This spectrum indicates that the molecular ion of the derivative is at a mass to charge ratio of 252; a value which is a measure of the compound's molecular weight. The appearance of a second peak in the spectrum at 31 mass units less than this value is consistent with the loss of a methyl ester grouping ($-OCH_3$). The base peak of 100, seen at a mass to charge ratio of 163, was indicative of the most likely molecular fragment. Peaks were seen at 76 and 77, and

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Table Two. The methyl ester of the unknown compound was prepared as previously described. Samples of the purified derivative were freed of solvent in vacuo and were submitted for mass spectral analysis.

The peaks which were observed and measured are shown. Only those peaks having a relative intensity of 10 % or greater are shown. These fragments represent the most likely cleavage products, and are the most helpful in determination of structure.

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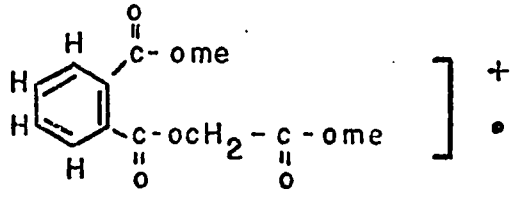
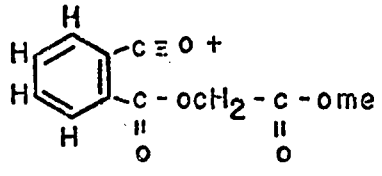
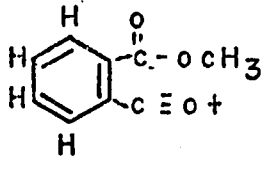
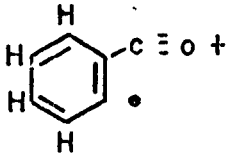
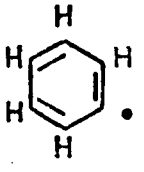
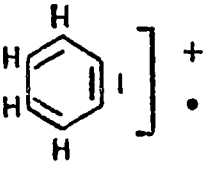
TABLE TWO

Mass spectral fragmentation pattern of the methyl ester of the unknown compound.

Mass to charge ratio	Relative Intensity	Possible structure
252	10 %	
221	12.5	
163	100	
104	10	
77	13	
76	13	

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were consistent with two substituents on a benzene ring. (This information confirms the NMR finding of four ortho aromatic protons.) A peak at 104 is consistent with a carbonyl attached to a substituted benzene ring and the peak at 163 could be a substituted benzene to which is attached methyl ester and a second carbonyl. The 58 mass unit difference between 221 and 63 can be accounted for by a methylene group located between a carbonyl and a carboxyl ester. Once again this assignment conforms with the NMR data which indicated methylene protons adjacent to a strong electron withdrawing group.

The most likely structure of C, H, and O which fits these criteria is the methyl ester of the molecule, glycolyl phthalate which is shown in figure six. Accurate measurements of the molecular weight of the methyl ester of the unknown compound were made using high resolution mass spectrometry, and this value was compared to the calculated molecular weight for the dimethyl ester of the molecule shown in figure 6. Table 3 indicates that the agreement between the two values is quite good and is confirming evidence for the validity of the structural assignment.

Absolute confirmation of structure was obtained by the chemical synthesis of glycolyl phthalate from phthalic anhydride and glycolic acid. After this material was purified and treated in a manner similar to biologically derived material, the IR spectra of the two methylated compounds was measured. They are shown in figure seven and it can be seen that they are identical. Thus, confirmation of structure is proven by synthesis.

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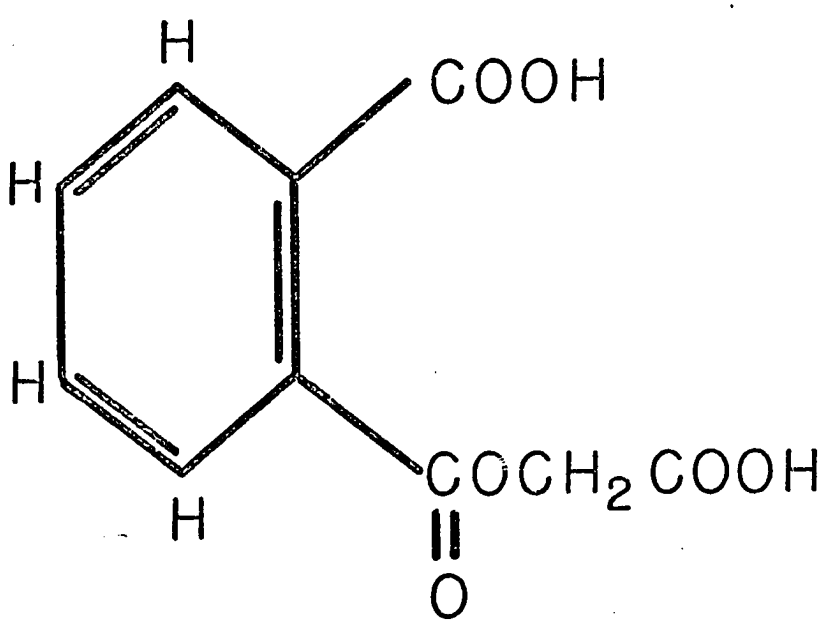
FIGURE SIX



Glycolyl Phthalate (GP)

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Table Three. The molecular weight of the methylated unknown compound was determined by high resolution mass spectrometry. This procedure determines the mass to charge ratio of the molecular ion, and does so with four decimal place accuracy.

All combinations of C, H, N, and O which could have a molecular ion in the 252 Dalton range were calculated and compared to the measured value. Only the empirical formula $C_{16}H_{12}O_4$ was sufficiently close to this value. This formula corresponds to the structure assigned to the methylated derivative of the unknown compound.

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TABLE THREE

HIGH RESOLUTION MASS SPECTRAL MEASUREMENT

OF THE MOLECULAR ION OF THE METHYL ESTER

OF THE UNKNOWN COMPOUND

CALCULATED MASS OF PROPOSED STRUCTURE	252.0634
MEASURED MASS	252.0636

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Figure 7. Pure glycolyl phthalate was synthesized as described in methods. Similarly, biologically derived samples of the unknown compound were prepared. The materials were methylated with diazomethane and the IR spectra were measured in Perkin Elmer 21 infrared recording spectrophotometer.

Samples were dissolved in acetone, and were spread on flat sodium chloride windows. After the solvent had evaporated, the spectra were determined with air as the reference.

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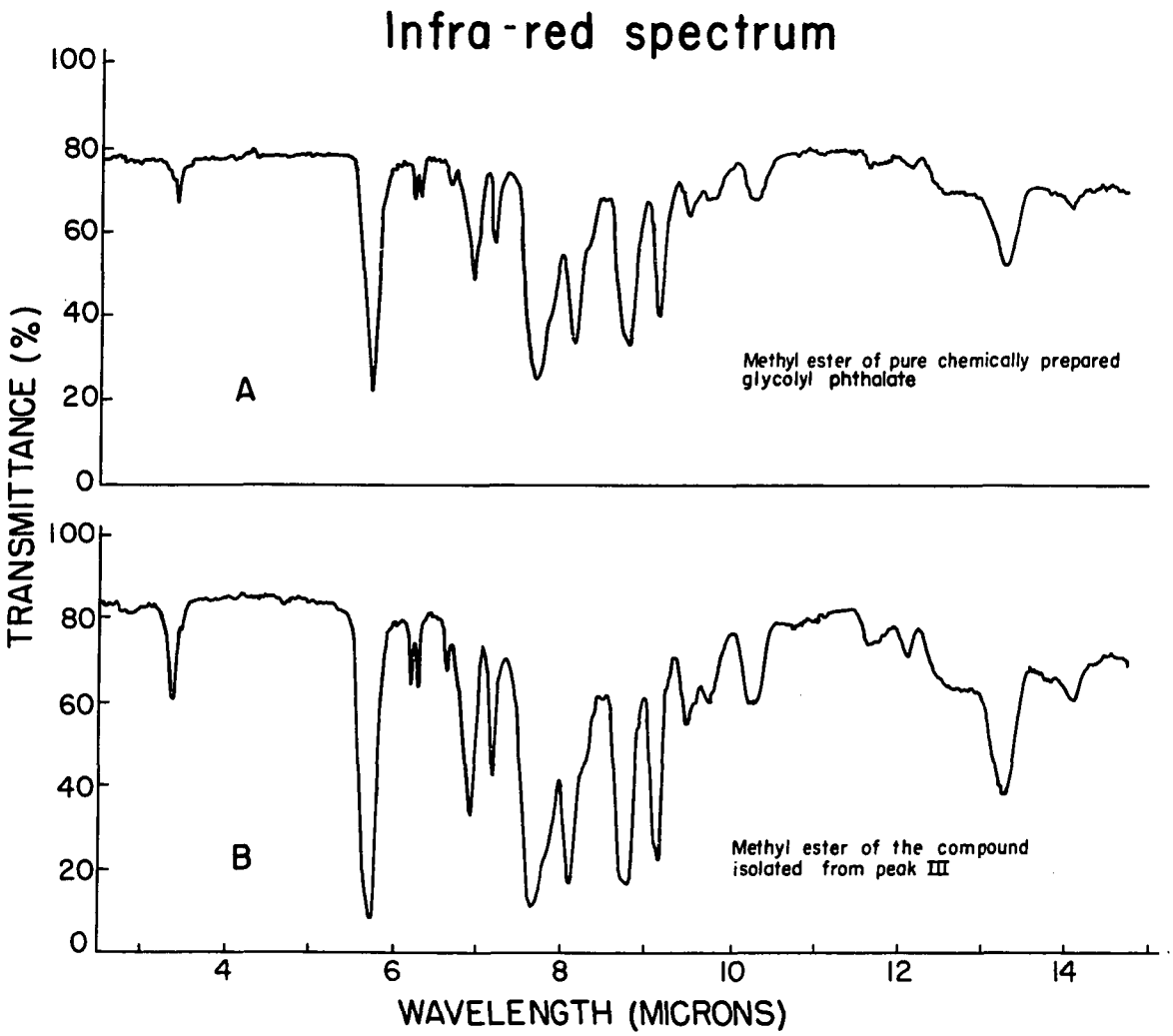

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C. Source of Glycolyl Phthalate

The presence of a phthalate compound in the perfusion fluid of the isolated, rat liver was a strange finding. These chemicals are not normal constituents of living organisms, but it is well known that esters of phthalic acid are used as plasticizers of polyvinyl chloride tubing and containers (Noeller,1965). Therefore, it was suspected that the appearance of GP might be a result of contamination of perfusate by a plasticizer from the PVC tubing used in the perfusion apparatus.

The manufacturer of the tubing (Norton Co., Akron, Ohio) was contacted and the company identified the plasticizer used in the tubing, medical surgical grade Tygon (S-22-1), as butyl glycolylbutyl phthalate (BGBP) the dibutyl ester of glycolyl phthlate. A pure sample of BGBP was obtained from the Monsanto Chemical Co. and was compared by thin layer chromatography to organic extracts of the tubing. BGBP, extracted from the tubing,was identical to samples of pure BGBP, thus confirming the tubing manufacturer's identification.

Organic extracts of perfusate that had circulated in the apparatus in the absence of a liver were subjected to thin layer chromatography. The plasticizer BGBP was found to be present in these solutions after a four hour circulation in the apparatus. On the other hand, when an isolated liver was present in the perfusion system, BGBP could not be found; under these conditions GP was identified as indicated above. Thus the evidence was quite strong that the tubing contributed BGBP, that this plasticizer was metabolized by the liver, and that the product of

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this metabolism, glycolyl phthalate, then accumulated. In order to prove this suspicion, a liver perfusion was performed under conditions which rigorously excluded the presence of any phthalate ester plasticizers, and similarly, another experiment was performed in which a large excess of BGBP was added exogenously to the perfusate. The results of these experiments are shown in figure eight; also shown in this figure is the amount of GP which results from the normal perfusion of a rat liver.

Experiment C, in which the plasticizer was excluded by the use of ether-extracted gum rubber tubing, demonstrates that no GP is detected after 4.5 hours of perfusion. Under normal circumstances, experiment B indicates that there is accumulation of GP in the perfusate when BGBP plasticized tubing is used. Further, it is clear that the exogenous addition of an amount of BGBP results in an increased accumulation of GP after 4.5 hours of perfusion as shown in experiment A. From these experiments, it can be concluded that the source of GP was BGBP extracted from the tubing by the perfusate and acted on by the liver, possibly through the action of carboxyl esterases known to be present in that tissue (Dixon and Webb, 1964; Lauwerys and Murphy, 1969). Thus, the accumulation of GP results from the closed system that the isolated perfused liver represents. In an open system such as is represented by the living organism, renal clearance of this organic acid, GP may be possible.

Therefore, the intact animal was tested for its ability to metabolize and excrete a metabolite of phthalate ester, BGBP. Two rats were given injections of the plasticizer and urine was collected and analyzed qualitatively for its GP content. As expected from the perfusion

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Figure 8. Perfusion experiments were done as outlined previously. The content of glycolyl phthalate was determined as follows. In experiment A, after extraction of the plasma with chloroform at neutral pH to remove lipid-soluble material, a portion of the remaining aqueous phase was fractionated by thin-layer chromatography. The spot corresponding to GP was scraped from the plate, the GP was eluted from the powder into water, and its concentration was determined by its absorption in the ultraviolet. In experiment B, the plasma sample was acid-precipitated. The increase in absorbance at 280 nm of the neutralized, acid-soluble supernatant fraction was taken as a measure of the amount of GP accumulating. In experiment C, only the 4-hour point was determined. This was done by fractionating the acid-soluble supernatant fraction of the plasma from the total perfusate on a Dowex-1 column. As indicated, no GP was detected.

A = Exogenous BGBP

B = BGBP plasticized tubing

C = Gum rubber tubing

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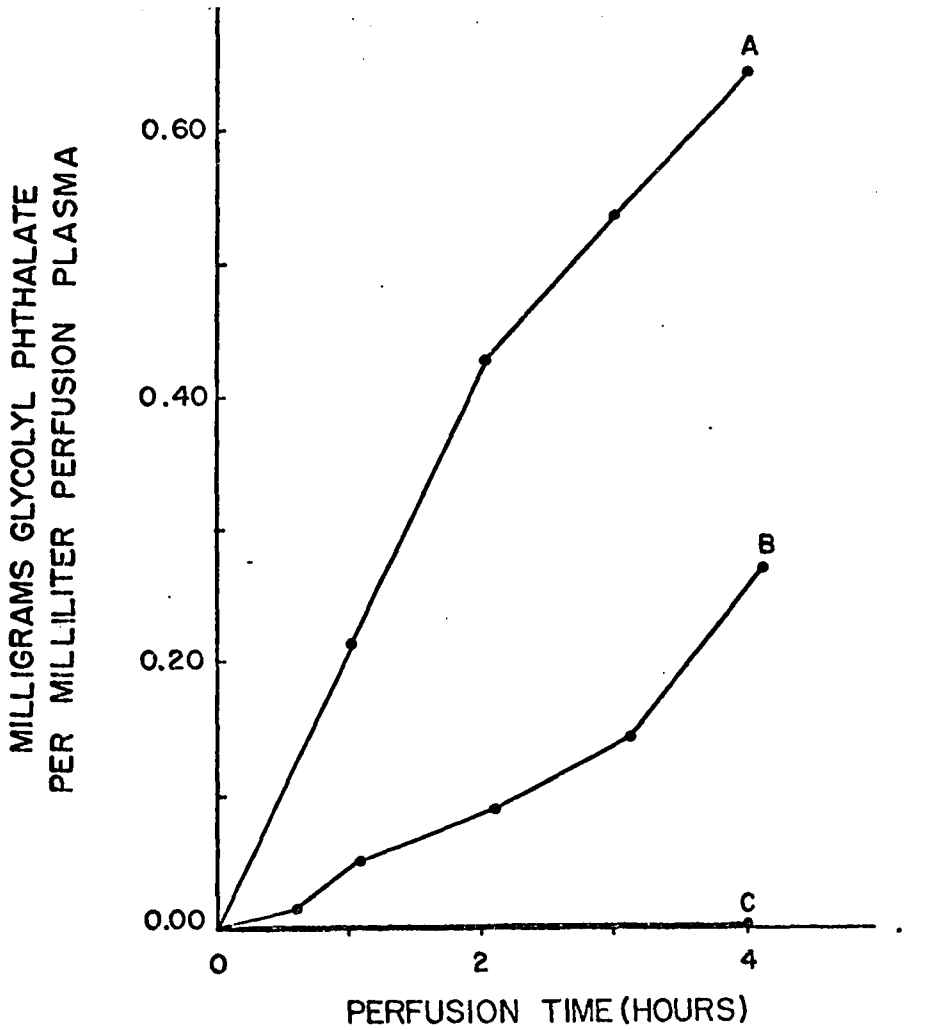
Time course of glycolyl phthalate accumulation in the isolated, perfused rat liver system.



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experiments, glycolyl phthalate was present in this urine.

D. Di-2-ethylhexyl Phthalate - Extraction and Disposition

A second phthalate ester plasticizer that is widely employed in PVC formulations is di-2-ethylhexyl phthalate (DEHP). Medical grades of DEHP plasticized PVC plastic are available as surgical tubing, catheters, and other medical appliances. Medical grade Tygon tubing, S-50-HL (Norton Co., Akron, Ohio) made of PVC and containing DEHP as the plasticizer was tested for extractability in the isolated perfused liver system. Samples of perfusate were circulated for 4 hours in the tubing (no liver present) and an organic extract of the perfusate was made. This extract was examined by qualitative thin layer chromatography, and it can be seen from figure nine that DEHP is present after the circulation of perfusate in the absence of a liver.

The isolated perfused rat liver preparation was employed to determine if the DEHP which is known to be extracted by the perfusate was metabolized. Such metabolism has been shown for BGBP where the metabolite found in the perfused liver system was GP. However, in the case of DEHP, the expected metabolite would be phthalic acid, and should be readily detectable by anion exchange chromatography or thin layer chromatography. However, in experiments utilizing PVC tubing plasticized with DEHP, no phthalic acid could be detected in acid soluble extracts of perfusate plasma, liver, or bile after 4.5 hours of perfusion. Further, when organic extracts of the perfusate from a liver perfusion

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Figure 9. Perfusion fluid was freshly prepared, and one-half of the perfusate was circulated through tubing of the liver perfusion apparatus (Tygon S-50HL). The circulation time was five hours at 37°C. During the time of circulation, the control sample of perfusate was incubated within the chamber but was stored in glass. The two samples were extracted with chloroform-methanol (see methods). The final lipid extract was spotted on 250 micron thick silica gel TLC plates on glass. They were developed in solvent saturated tanks of methylene chloride, and the DEHP was visualized by the thymol regeant described by Pereboom (1960). The amounts of the extract which were spotted was approximately 5% of the total lipid present.

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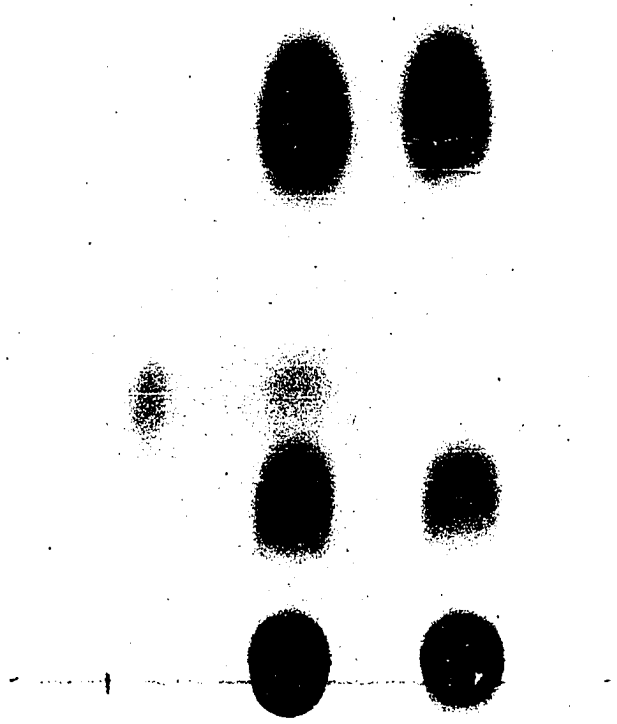
THIN LAYER CHROMATOGRAM

OF PERFUSION FLUID



DEHP | Circulated Perfusion Fluid | Non-Circulated Perfusion Fluid

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experiment were examined by TLC, no DEHP could be found, a result which indicated that the liver may have removed the plasticizer.

When whole liver homogenates were incubated with DEHP, ether extractable phthalic acid could not be found although experiments under similar conditions resulted in the formation of GP from BGBP. Thus, either of two conclusions are possible: first, that DEHP is metabolized by the liver to a compound other than phthalic acid, or second, that DEHP is not metabolized but is removed from the perfusate.

This latter possibility was tested in the following experiment. The PVC tubing of the apparatus was replaced by tubing of silicon rubber which does not contain plasticizers. An amount of DEHP was added to the perfusate as a sonicated emulsion in bovine serum albumin, and the mixture was allowed to circulate in the apparatus. Immediately prior to the installation of the liver within the system (time zero) and at 0.5 hour and 4.5 hours of perfusion, aliquots of perfusate were removed and assayed for DEHP content. After the 4.5 hour a sample was taken, the liver was saline perfused to remove all intravascular blood and assayed for the hepatic concentration of DEHP.

Figure ten shows the result of this experiment. The data indicates that DEHP disappears very rapidly from the perfusate. Furthermore, after 4.5 hours of perfusion 91% of the plasticizer can be recovered in the liver, indicating that some specific and highly efficient uptake process has occurred. Because the livers had all been perfused with saline at the termination of perfusion, the amounts of DEHP recovered must have

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Figure 10. Liver perfusion experiments were performed with silicon rubber tubing rather than PVC as previously used. The perfusion fluid was prepared as described in methods but an amount of DEHP was added to the BSA (bovine serum albumin solution) and this mixture was sonicated. The amount of DEHP added was such that the final perfusate content of DEHP would be 70 - 80 μg/ml.

The perfusate was circulated in the apparatus and samples were withdrawn at 0, 0.5, and 4.5 hours. The amount of DEHP present at time zero was determined by measurement of the concentration times the volume of the system. The DEHP in samples of perfusate and liver are expressed as a percentage of this value.

The perfusion was done as described in methods except that the livers were saline perfused at the end of the experiment. DEHP analysis was performed as described.

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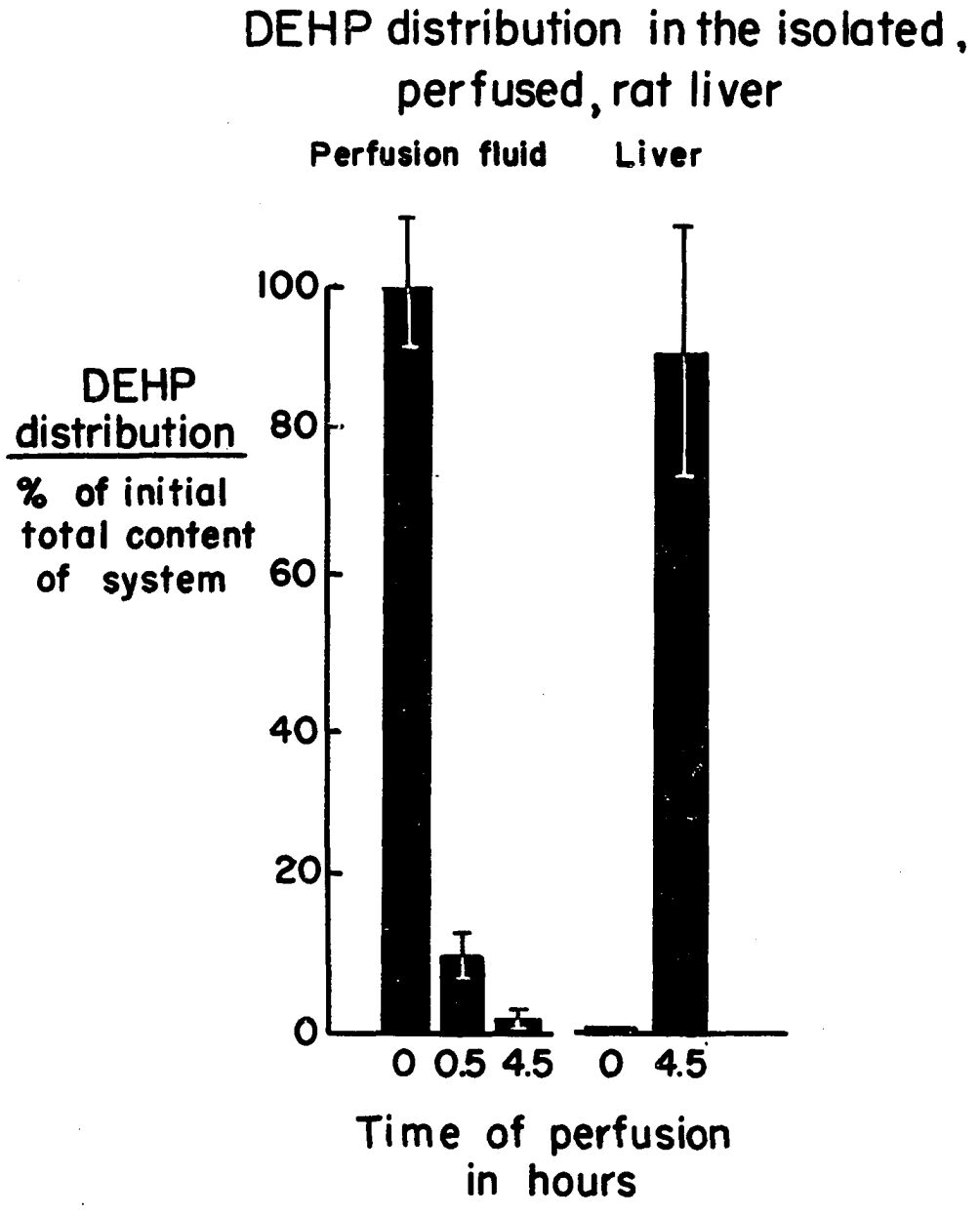

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been intracellular or membrane bound and could not represent an intravascular component. In addition, no phthalic acid could be detected in the perfusate from these experiments and thus it is concluded that DEHP administered intravenously to the isolated perfused rat liver is accumulated within that organ in an essentially unchanged form.

This apparent lack of metabolism is not in agreement with a previous report in the literature by Shaffer et al (1945). These authors stated that DEHP is metabolized when given orally to the rat, and the product of metabolism, phthalic acid, could be recovered from the urine of the intact animal. Therefore, their experiments were repeated here to determine if the isolated perfused liver is somehow different from the whole animal.

Four groups of two rats each (200 - 250 gram range) were housed in cages that permitted the daily collection of urine. The treatment period was eight days, and during this time, group one served as the control, eating only the control diet (Nutritional Biochemical Co., Cleveland, Ohio). The second group received the DEHP mixed in the diet at a level of 1% while the third group received a single dose of DEHP, intraperitoneally. A fourth group received a single dose of phthalic acid intraperitoneally. Urinary phthalic acid was measured gas chromatographically after the pooled daily urine samples of each group had been acidified, and ether extracted. Ether extracts were treated with diazomethane to convert the organic acids present to methyl esters. The results of this experiment are shown in table four.

It can be seen from the data that measurable amounts of phthalic

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Table Four. Four groups of two rats each (200 grams) were housed in cages that permitted the collection of urine. The groups were all fed a standard synthetic diet (Nutritional Biochemicals Co., Cleveland, Ohio), and they were treated as follows. Group one was fed only the diet. Group two received a diet which contained 1% DEHP. Group three received a single injection of 1 ml of DEHP given as the pure liquid. The injection was made intraperitoneally. Group four received an injection of phthalic acid (38.1 mgs/rat). All groups were given tap water ad libitum.

Urine was collected for eight days. The pooled daily urine samples were extracted as described in methods and the phthalic acid content was measured gas chromatographically. The numbers presented represent the total amount of phthalic acid detected divided by the number of rats in each group (2). Total food intake was determined, and the food consumed by each group is expressed as a percentage of the food consumption of the control group.

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TABLE FOUR

METABOLISM OF DEHP TO PHTHALIC ACID

GROUP	EIGHT DAY TOTAL URINARY PHTHALIC ACID	FOOD CONSUMPTION % OF CONTROL
1. CONTROL DIET	3.1 mgs	100%
2. DEHP DIET (1% w/w)	2.3	85
3. DEHP ip (1 ml)	2.4	61
4. PHTHALIC ACID ip (38.1 mgs)	35.1	112

acid are present in the urine from control animals, but in animals treated with DEHP, no increase in the urinary excretion of phthalic acid is seen to occur. The slight decrease in both cases (group 2 and 3) was associated with a decrease in food consumption, and thus, the urinary phthalic acid of control animals could have arisen from some phthalate present in the diet. Because of the almost total recovery of phthalic acid in the urine of PA treated rats (92%), it can be presumed that PA *per se* is not metabolized *in vivo*. This result has been shown by Erickson (1965) and thus, any PA which resulted from the metabolism of DEHP should have been detected in these experiments.

These results and those of the isolated perfused liver are still at variance with the experiments of Shaffer *et al* (1945). However, certain methodological differences exist. In the experiments reported in this dissertation, samples of urine were acidified with HCl and extracted with ether. The extract was treated with methylating agent, and the methyl esters of the organic acids were measured by gas chromatography. However, Shaffer *et al* (1945) reported that this extraction procedure caused the formation of stable emulsions and thus they treated the urine with KOH, followed by boiling to dryness. Samples were then refluxed with nitric acid for four hours after which extraction was done. The phthalic acid was measured by a gravimetric procedure using lead salts.

It is possible that the pretreatment of the urine with hot base and hot acid could have converted a phthalic acid-containing metabolite of DEHP to PA. Therefore, an experiment was performed with human urine,

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and both methodologies were applied. The result that was obtained is shown in table 5 and it can be seen that the increase in urinary "phthalic acid"* content after Shaffer hydrolysis does indicate that considerable conversion had taken place. The significance of this amount of "PA" in human urine will be discussed in a later section.

E. Di-2-ethylhexyl Phthalate - Tissue Distribution After iv Dose and Rate of Disappearance after ip Injection

It has been shown in experiments utilizing the isolated perfused liver that intravenously administered emulsions of DEHP are accumulated within that organ. The experiments with the intact animal have shown that oral and intraperitoneal doses of DEHP are not metabolized to urinary phthalic acid per se. A further experiment was performed to determine the organ distribution of the plasticizer in the rat following intravenous administration. The results obtained are shown in table six.

It can be seen from the data that both lung and liver contain large amounts of the plasticizer as does the residual carcass. However, on a per gram basis, the highest concentration is present in the lung while liver and spleen have lower concentrations. The concentration of DEHP found in kidney, heart, gut, brain, testicle, and carcass indicates that the accumulation of amounts of DEHP in tissue is not a function of simple capillary filtration but rather is specific for those tissues associated with recticulo-endothelial function. The amount of the plasticizer

---

* "Phthalic acid" indicates total PA content after Shaffer hydolysis.

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Table Five. Human urine was examined for its content of phthalic acid. Samples were acidified and extracted with diethyl ether as described in methods. The phthalic acid was determined by gas chromatography of the methylated derivative, dimethyl phthalate.

Another aliquot of the same urine was hydrolyzed according to the methods of Shaffer et al (1945). The amount of phthalic acid present after hydrolysis was measured by GC as described in methods. In both methods, standards of phthalic acid were included in samples of urine so that the total recovery could be assessed. The final measured levels of PA were corrected for these recoveries.

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TABLE FIVE

DIFFERENCES IN URINARY PHTHALIC ACID BY TWO METHODS

	URINARY LEVEL
JAEGER METHOD	52 µg/100 ml
SHAFFER ET AL	480 µg/100 ml

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Table Six. An aqueous emulsion of DEHP was prepared by sonication of an amount of the plasticizer in a solution of 4% bovine serum albumin. The final concentration of this solution was 40 mgs/ml. Two rats (200 gms) were given one ml injections via the dorsal penis vein. Light ether anesthesia was used during the injection.

After 24 hours, the animals were killed by decapitation and shed blood was collected in a heparinized container with minimal blood loss. The organs were removed, weighed and frozen. The feces and carcasses were homogenized in saline and an aliquot was removed for analysis. All samples were lyophillized, and assayed as described in methods.

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TABLE SIX

INTRAVENOUS DEHP

RAT TISSUE LEVELS AFTER 24 HOURS

TISSUE	CONCENTRATION mg/gm or mg/ml	MGS. RECOVERED/ TISSUE	% OF INJECTED DOSE
LIVER	1.6	21.82	27.3
LUNG	8.18	21.26	26.6
CARCASS	0.06	16.66	20.8
SPLEEN	1.89	2.65	3.3
KIDNEY	0.08	0.26	0.3
HEART	0.08	0.14	0.2
GUT	<0.01	0.12	0.2
BLOOD	<0.01	0.03	<0.01
BRAIN	<0.01	0.01	<0.01
TESTICLE	<0.01	0.01	<0.01
FECES	<0.01	0.334	0.4

TOTAL DEHP RECOVERED: % OF INJECTED DOSE 79.1

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found in the carcass may have resulted from uptake by tissues such as lymph nodes and bone marrow, but these do not represent a significant portion of the whole carcass. Thus the DEHP concentration in the carcass is quite low relative to liver and lung.

An amount of DEHP was detected in the feces but this amount was small relative to the total dose injected. At present, it is not known why DEHP is present in this fraction since such a molecule would not normally be excreted by a biliary route in unaltered form. In any case, the presence of DEHP in this fraction does not represent a significant route of disposition.

This experiment indicated that the total recovery of DEHP from the whole animal was 79% of the injected dose after intravenous administration. Although no metabolism of the plasticizer to urinary phthalic acid could be demonstrated in this or earlier experiments, it may be possible that the disappearance with time of an injected dose of DEHP is representative of metabolism. Thus the differences between the results reported by Shaffer et al (1945) and the apparent lack of metabolism of DEHP reported here could partly be explained on the basis of methodology (Section D, table five).

Injections of DEHP were given intraperitoneally to rats, and at intervals after injection, animals were killed and the entire carcass extracted. Feces measurements were not made. The results of these experiments are given in figure eleven. The dose of DEHP given was 500 mgs/kg as aqueous emulsion in 3% acacia. The amounts of DEHP recovered are expressed as a percentage of the injected dose.

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Figure 11. Seven rats were given DEHP intraperitoneally as an aqueous emulsion of the plasticizer in 3% acacia/saline. The emulsion was prepared by sonication. The dose given was 500 mgs/kg.

At the times stated, the animals were killed by asphyxiation in a saturated ether atmosphere. The whole animal was homogenized in a one gallon Waring blender, and the final homogenate concentration was 10%. The mixture was filtered through acetone washed gauze, and an aliquot was removed.

DEHP analysis of this extract was done as described in methods.

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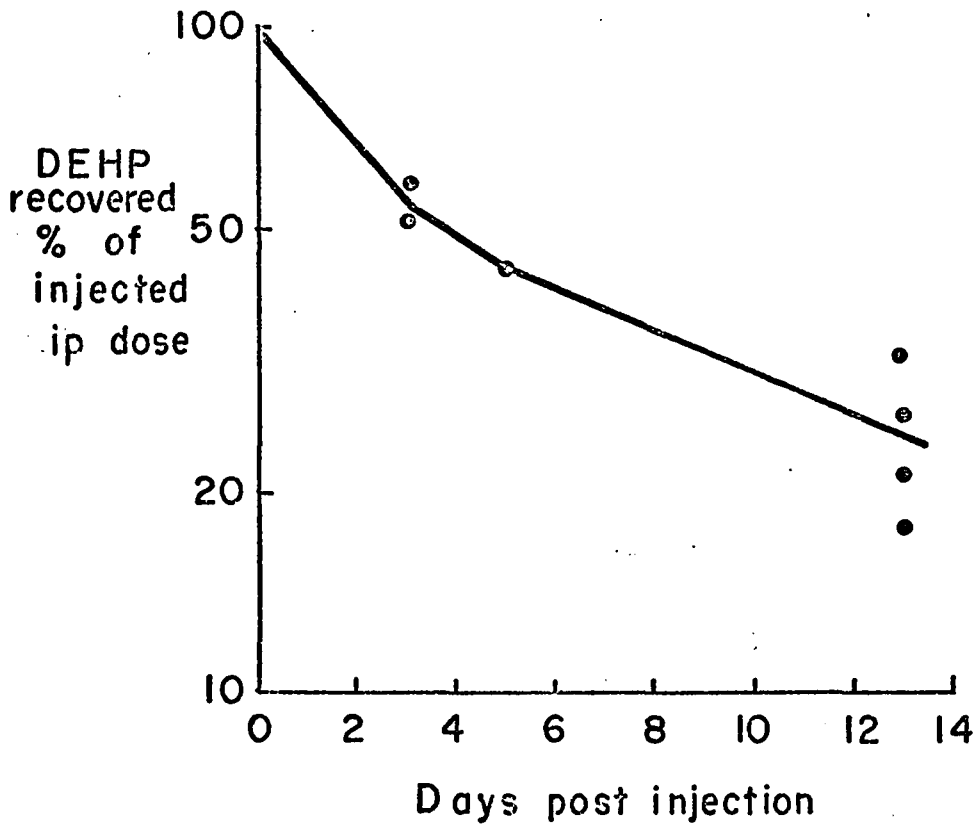
DEHP disappearance after intraperitoneal administration



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It can be seen from these results that DEHP does disappear from the rat at a very slow rate. However, this slow disappearance may not represent the rate of metabolism but rather may be a measure of the slow absorption of the lipid from the peritoneal cavity and its subsequent metabolism. When a smaller dose of DEHP was given iv to rats (previous experiment), the 79% recovery after 24 hours is consistent with the disappearance curve seen after ip administration (figure eleven). In any event, the results are indicative of some disposition of DEHP, and it could be concluded that this phthalate ester plasticizer is metabolized by the rat but only very slowly when administered under these circumstances. However, any metabolism that does occur appears to involve conversion of DEHP to a phthalate containing metabolite other than urinary phthalic acid *per se*.

F. Extraction of DEHP from Plastic Medical Devices By Human Blood and Blood Fractions

The experiments described have shown that the contamination of blood by DEHP is a consequence of that fluid having been circulated in tubing plasticized with DEHP. Therefore, experiments were performed with saline, synthetic plasma (4% bovine serum albumin), and rat blood which had been diluted with the plasma albumin solution (perfusion fluid).

The various solutions were circulated in medical-surgical grade Tygon tubing (S-50-HL, Norton Co., Akron, Ohio) for a period of five hours at 37°C. In each case, fifty milliliters of fluid were used. It can

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be seen from figure twelve that saline does not extract any plasticizer in excess of the blank solution. However, the proteinaceous albumin solution extracts 0.7 mgs of the plasticizer. A mixture of blood and albumin extracts significantly more plasticizer, 2 mgs, and this result indicates that the increased extraction of plasticizer by solutions which contain blood may be due to the presence of some factor other than protein, possibly lipid.

Tubing of polyvinyl chloride plastic is used for open heart surgery (cardio-pulmonary bypass) and for chronic hemodialysis. Therefore, the hypothesis was tested that human blood, like rat blood and bovine serum albumin solutions would extract the plasticizer from these types of medical tubing. Units of blood (500 ml) were obtained from the Johns Hopkins and Baltimore City Hospitals blood banks. These units were circulated in the hemodialysis apparatus (HD) and in the tubing of the heart-lung machine (CPB). The HD experiment required two units (1000 ml of blood) while the CPB experiment used four units (2000 ml of blood). The circulations were done at 37°C and the duration of each experiment was slightly different, 5 hours for CPB tubing, and 8 hours for HD tubing, but did approximate normal conditions of use.

The results of this experiment are shown in table seven. It can be seen from this table that there is a significant amount of DEHP plasticizer present in the blood before it is put into the apparatus. In the case of the CPB experiment, little increase in DEHP was seen over the five hour period. In the hemodialysis experiment, some increase

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Figure 12. Fifty milliliters of each solution were circulated in freshly washed samples of Tygon S-50-HL (Norton Co.). The lengths of tubing were equivalent to that used in the liver perfusion system. The time of circulation within the perfusion apparatus was five hours at 37°C.

All samples were lyophillized and extracted for DEHP as described in methods. Analysis of DEHP was done by gas chromatography as described.

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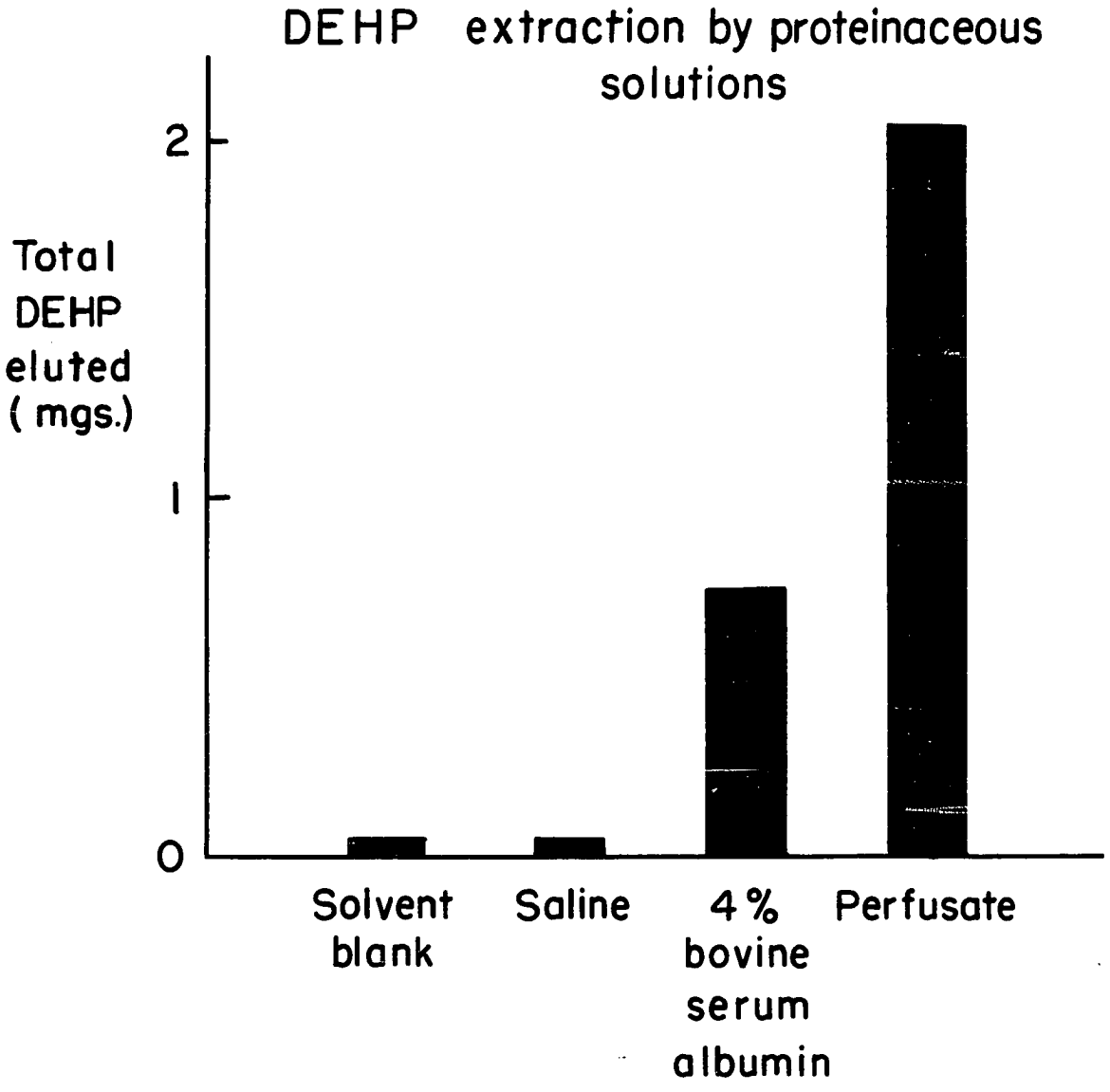

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Table Seven. Blood was obtained from the Johns Hopkins Hospital and Baltimore City Hospitals blood banks. All units had been stored under blood bank conditions for 22 days and were no longer suitable for human transfusion. The anticoagulant used was ACD-NIH formula A.

The hemodialysis experiment was set up as it is normally employed. ACD blood was further treated with heparin, and the appropriate amount of dialysis fluid was put into the tank. Circulation was for an eight hour period at 37°C.

The cardio-pulmonary bypass tubing was connected in series, and conditions were arranged such that all blood in the system flowed in a closed circle. These conditions approximated the actual conditions of use. Circulation of the blood was for five hours at 37°C.

Analysis was done on lyophillized blood samples as described in methods.

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TABLE SEVEN

EXTRACTION OF PLASTICIZERS FROM TWO TYPES OF MEDICAL DEVICES

MEDICAL DEVICE	HOURS OF CIRCULATION	PLASTICIZER CONCENTRATION mg/100 ml Blood	
		DEHP	DEHA
TRAVENOL HEMO-DIALYSIS UNIT	0	7.75	0
	8	9.24	2.14
TRAVENOL CARDIO-PULMONARY BYPASS UNIT	0	5.04	0
	2.5	5.28	2.34
	5	5.70	4.99

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in DEHP did occur, and this may have been due to the hemoconcentration that occurred in the blood as it was continually exposed to hypertonic bathing fluid. Although the hematocrit of this blood (HD) was adjusted with saline during the experiment, the temporary increase in concentration of blood proteins and lipids may have resulted in increased extraction of DEHP in this case.

It should be noted that a second plasticizer, later identified as di-2-ethylhexyl adipate (DEHA), increased in concentration during both experiments. This second plasticizer was found to be present in the tubing of these two systems (Travenol Artificial Organs, Baxter Laboratories, Morton Grove, Ill.), but it was never found in PVC blood storage bags. The rate of increase of this second plasticizer indicated that if the DEHP content of blood bank-stored blood had been low at the beginning of the experiment, it too might have increased with time.

The finding that blood bank stored blood contains DEHP was interesting. Human blood is drawn in plastic bags of PVC but it is stored at $4^{\circ}C$. It was assumed that the DEHP content would be low in this blood but apparently, the relatively long storage time of 21 days is sufficient for extraction to occur, even at this temperature. Therefore, units of blood were obtained from the Johns Hopkins blood bank after various times of storage, and these were analyzed for their DEHP content. Similarly dog blood was drawn and stored in these same type blood bags (Fenwal JA-2C Blood Pack, Baxter Laboratories, Morton, Grove, Ill.). All units were analyzed for their DEHP content as described in mehtods.

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The results of these experiments are shown in figure thirteen. It can be seen that with increasing time of storage, an increased amount of DEHP may be recovered from the stored blood. The slope of the linear regression equation is 0.25 mgs/100 ml/day. From this value, a whole body exchange transfusion in a 70 kg man using twenty day old blood would result in the intravenous administration of approximately 300 mgs of DEHP or a dose of 4 - 5 mgs/kg.

The unit of human blood obtained at 14 days of age was fractionated to determine the concentration of DEHP in the individual fractions. This was done to ascertain the fraction of blood possibly responsible for the extraction of DEHP. Blood was separated into plasma and red cells; the red cells were washed with saline to remove traces of plasma. The plasma was further separated into two sub-components, that portion with a density greater than 1.21 and that portion of density less than 1.21. The fraction of density less than 1.21 is known to contain low desity lipoproteins and lipid droplets. Thus DEHP droplets with a density of 0.99 would float under these conditions. Any plasticizer recovered from the heavier fractions would represent plasticizer bound to protein or membranes.

The results of this experiment are shown in figure fourteen. It can be seen that the bulk of the recovered plasticizer is present in the lipoprotein or buoyant fraction. However, DEHP is present in the $d > 1.21$ fraction and the washed red cell fraction. These levels are likely to represent bound plasticizer. It can also be seen from the figure

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Figure 13. Samples of stored human blood were obtained from the Johns Hopkins Hospital and Baltimore City Hospitals blood banks. In addition, standard ACD blood bags were filled with dog blood. All bags were sampled at the times indicated. The data points for human blood represen different blood samples which were stored for the indicated periods of time. The data points for the dog blood represent three different units of blood. A single unit was assayed after less than one day of storage and then discarded. The other two units were sampled repeatedly using asceptic techniques at the times specified. The DEHP measurements of the two units of dog blood were averaged and only the mean value is shown in this figure.

Aliquots were removed from the bags and were assayed as described. The results were plotted as a straight line using the least squares line of best fit.

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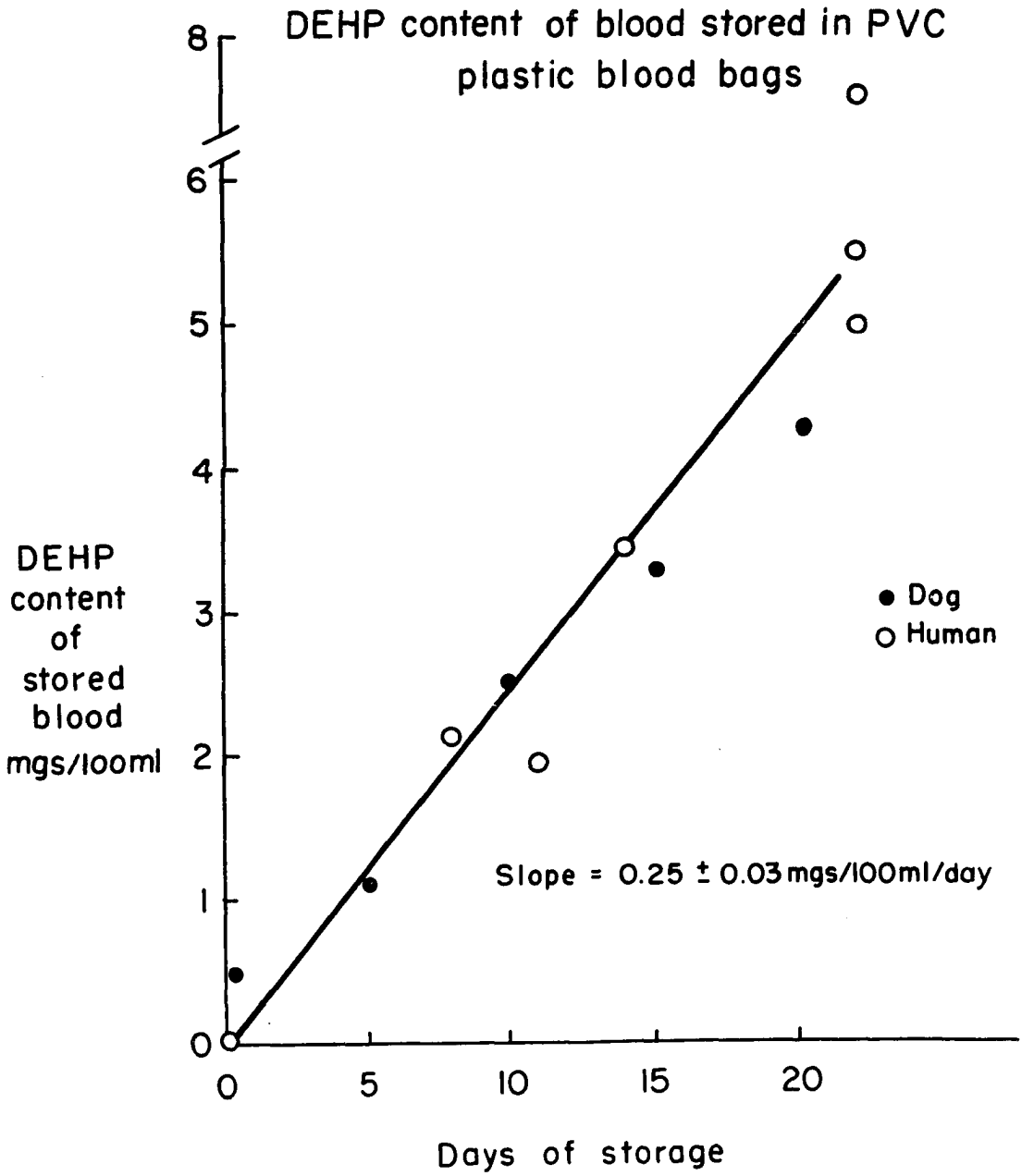

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Figure 14. An aliquot of blood from a single unit of fourteen day old human blood was separated into two fractions, packed red cells and plasma. The red cells were separated from the bulk of the plasma and were washed successively with saline. This fraction was set aside and frozen.

The density of the plasma was adjusted with sodium bromide so that the final density was 1.21. The sample was centrifuged in Spinco Model L using a #30 rotor. The samples were spun for 40 hours at 25,000 rpm.

Fractions of $d<1.21$ were isolated by cutting off the top portion of the tube. The two samples were frozen, and all samples were lyophillized. Analysis of DEHP was done as described.

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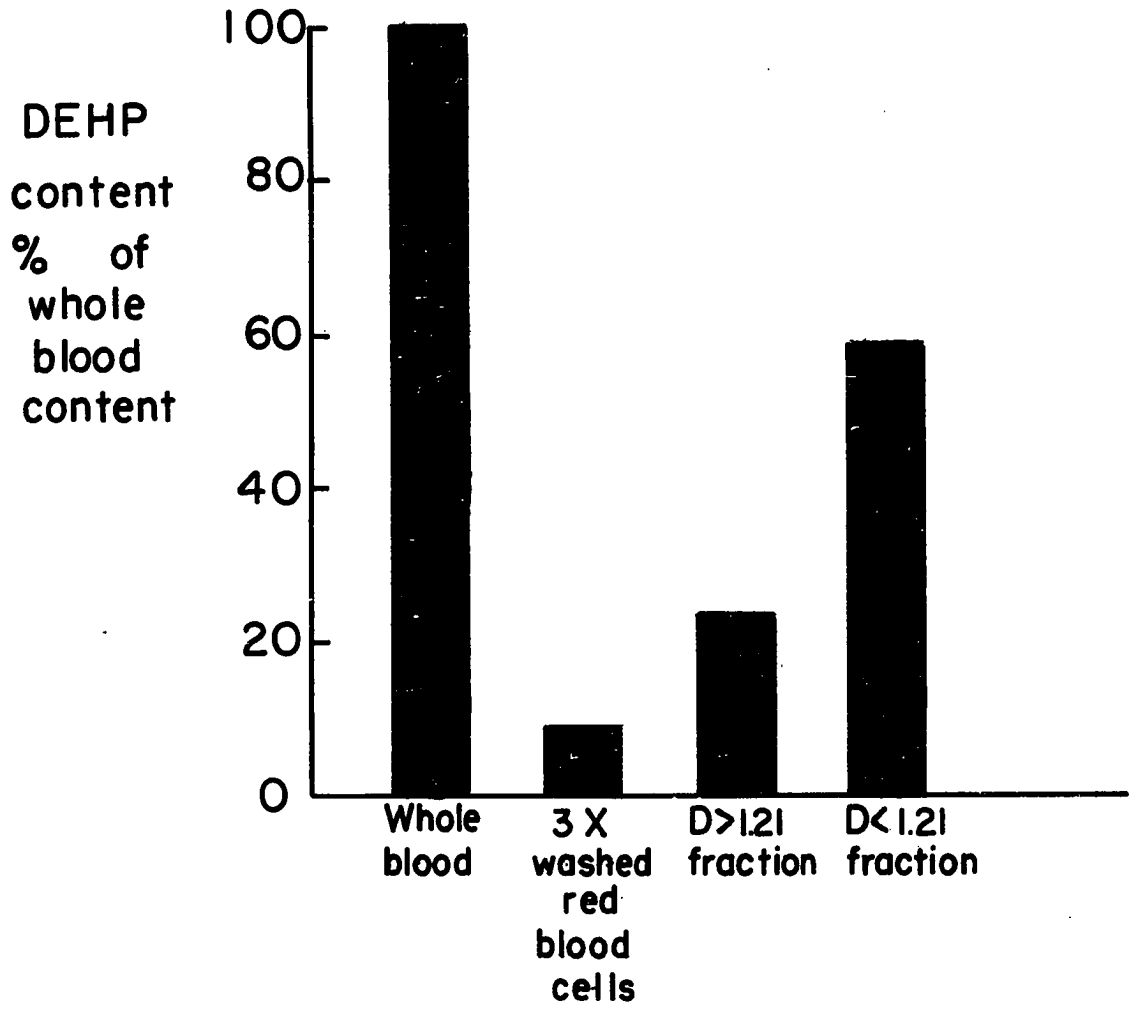
DEHP recovery from 14 day PVC stored human blood, and blood fractions.



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that recovery of DEHP from these three fractions is approximately 91. 3% that of whole blood.

G. Di-2-ethylhexyl Phthalate Accumulation in Human Tissue

With the demonstration of the presence of certain plasticizers in blood stored in plastic bags or circulated through plastic tubing, it became of considerable interest to determine the presence of such compounds in the tissues of humans who had received transfusions of plastic-stored blood or who had undergone surgical procedures in which their own blood was exposed to plastic tubing. Furthermore, it was of interest to determine the presence of DEHP in the adipose tissue of normal humans who had not directly received DEHP-contaminated blood, but who might have demonstrable amounts of the plasticizer due to the ingestion of fatty and proteinaceous food products that had been stored or transported in PVC plastic.

Samples of human tissue were obtained at autopsy from individuals who had died after receiving blood transfusions or who had undergone open heart surgery. Similarly, tissues were obtained from individuals who had died as a result of accidents or suicide. The latter individuals had received no known blood transfusions. The results of these analysis in terms of the amounts of DEHP recovered per gram of dry tissue weight is given in table eight. It can be seen from these data that amounts of DEHP can be detected in various tissues from certain individuals. Further, no DEHP could be found in the body fat of normal individuals who had

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Table Eight. Samples of human tissue (lung, liver, spleen, etc.) were obtained at autopsy from patients who had died following open heart surgery or blood transfusion. Similarly, samples of body fat from normal persons who died after accidents were obtained from the medical examiner's office.

All tissues were analyzed as described in methods. The amounts of DEHP present in the tissue is based on the total recovered per gram of dry weight. Samples of blood from three individuals were analyzed and the DEHP content of these are expressed as mgs per 100 ml of whole blood.

Samples that are listed as ND indicate that the amount of DEHP present in tissues was not different from the solvent blank of that experiment. Detection limits for the assay procedures were found to be five parts per million of dry tissue weight.

* DEHP dose was calculated from the known storage time of each unit transfused and the previously determined DEHP extraction rate of 0.25 mgs/100 ml/day.

** Dry weight basis

NK = not known

—— = assay not performed

ND = not detectable

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TABLE EIGHT

DEHP CONTENT OF HUMAN TISSUES

PATIENT (Sex)	HISTORY	UNITS OF BLOOD	CALC* DEHP mgs.	DEHP CONTENT ** LUNG	LIVER	SPLEEN	ABD. FAT	BLOOD
				(µg/gm)				(mg/100 ml)
PR(F)	CPB	18	43.8	91.8	69.5	25.3	--	ND
GM(M)	HD	NK	NK	--	--	--	--	ND
AC(F)	Pancreatitis	13	NK	--	--	--	270	--
BB(M)	Aneurism	4	22.5	21.2	ND	5	--	--
CS(M)	Mult. Transf. Pancytopenia	NK	NK	20.8	--	--	ND	--
RD(M)	CPB	6	16.8	ND	ND	ND	--	--
RT(M)	GI Bleeding	31	NK	ND	ND	--	--	--
TH(M)	CPB	8	128	17.9	--	--	ND	--
WD(M)	Mult. Transf.	NK	NK	13.4	--	--	ND	--
HC(M)	CPB	20	NK	22.1	--	--	--	--
MC(F)	Pneumonia	2	NK	ND	ND	ND	--	--
JG(M)	CPB	14	NK	ND	ND	ND	ND	--
RC(M)	CPB	4	14	24.5	--	--	--	--
RM(M)	Gunshot	63	600	ND	ND	--	--	0.28
Seven accident victims who received no known blood transfusions (6 males and 1 female)				--	--	--	ND	--

not received transfusions (seven cases of accidental death).

In addition, blood samples were taken from two patients, GM (M) and PR (F), during the course of hemodialysis and open heart surgery, respectively. It was found that, although the blood of these individuals was being exposed externally to plasticized plastic tubing, samples of their systemic blood did not contain any trace of DEHP. It might be presumed that some organ in these individuals was efficiently removing the plasticizer from the circulatory system, much as the data for the isolated perfused rat liver would suggest. In fact, one of these two patients, PR (F), was on cardiac bypass and died after surgery. The data in table eight indicates that she had significant quantities of DEHP in her tissues.

It should be pointed out that not all individuals exposed directly to PVC during CPB had observable amounts of DEHP in their tissues (e.g., RD(M), JG (M)). Likewise, of these individuals who had received calculated amounts of DEHP from transfusions of stored blood, not all demonstrated the plasticizer in their tissues (e.g., RT (M), MC (F)). This observation is highlighted by the results seen in patient RM (M). This individual received 63 units of blood during a 24 hour period following a gun shot wound of the abdomen. In spite of an aortic graft, splenectomy and partial hepatectomy, massive hemmorrhage led to his death. By means of the recorded storage age of each unit of blood and with reference to the time-related rate of elution of DEHP from plastic blood storage bags (figure thirteen), it could be determined that this individual

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should have received over 600 mgs of the plasticizer. Immediately after death, a sample of peripheral venous blood was drawn and as is shown in the table, it did contain a measurable amount of DEHP (0.28 mgs/100 ml). However, when tissue was obtained at autopsy the following day, and subsequently analyzed, no DEHP could be found in lung or liver.

The reasons why, some individuals who have presumably received DEHP but do not demonstrate the presence of the plasticizer in their tissues, are not entirely clear at this time. One possibility to be considered is that such individuals are capable of rapidly metabolizing DEHP. It should be emphasized, however, that such a possibility is contrary to the evidence in the rat where the rate of DEHP disappearance is quite slow. On the other hand, species and individual differences in rates of metabolism of foreign compounds are not to be unexpected. These differences may be further altered by disease processes unique to certain individuals in this study.

H. Metabolism of DEHP in the Human

It has been shown that certain individuals receiving blood transfusions or undergoing procedures involving extracorporeal circulation of blood have measurable levels of DEHP in their tissues. Other persons, for reasons at yet unclear, are able to dispose of this foreign compound. Therefore, one individual was followed before and after successful open heart surgery. Urine from this patient was collected two days prior to surgery and for six days (including the day of surgery) following the

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surgery. This patient, RM (M) was on cardiac bypass for 1.5 hours and received 8 units of blood, which from knowledge of its duration of storage in plastic blood bags should have resulted in the transfusion of approximately 21 mgs of DEHP. Samples of urine from pre and post surgery were acidified with HCl and ether extracted after which the dry ether extracts were treated with diazomethane. They were then analyzed directly for PA (as the dimethyl ester) by gas chromatography.

The results are shown in figure fifteen (solid bars). Phthalic acid was found to be present in both pre and post surgery samples or urine. However, the amount of phthalic acid excreted in the urine during the six days following surgery (including the day of surgery) was calculated to be 3.7 mgs greater than might have been expected on the basis of the control amount excreted during the pre-surgical period. The bulk of this was excreted immediately following surgery. This amount of phthalic acid could account for only 8.7 mgs of DEHP, or less than half of the estimated 21 mgs DEHP received by the patient from transfusions alone. This amount of phthalic acid does not account for any removal of additional amounts of DEHP from the tubing of the cardio-pulmonary bypass apparatus by the patient's blood during the 1.5 hours of external circulation.

It has been shown by Shaffer et al (1945) that both rats and man are capable of metabolizing orally administered amounts of DEHP. As was mentioned previously (Section D), the methods that these authors employed differ markedly from those used in this dissertation. As was shown in table five with the urine of patient RM, there is a nine fold

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Figure 15. Urine was collected from patient RM for two days prior to open heart surgery and for the six days following surgery (day zero indicates the day of surgery). Samples of urine were acidified and ether extracted after which they were analyzed for phthalic acid as described in methods. Alternatively, urine samples were hydrolyzed by the method of Shaffer et al (1945), and were subsequently ether extracted after which the samples were treated with diazomethane. The phthalic acid content was measured directly by gas chromatography of the dimethyl phthalate derivative.

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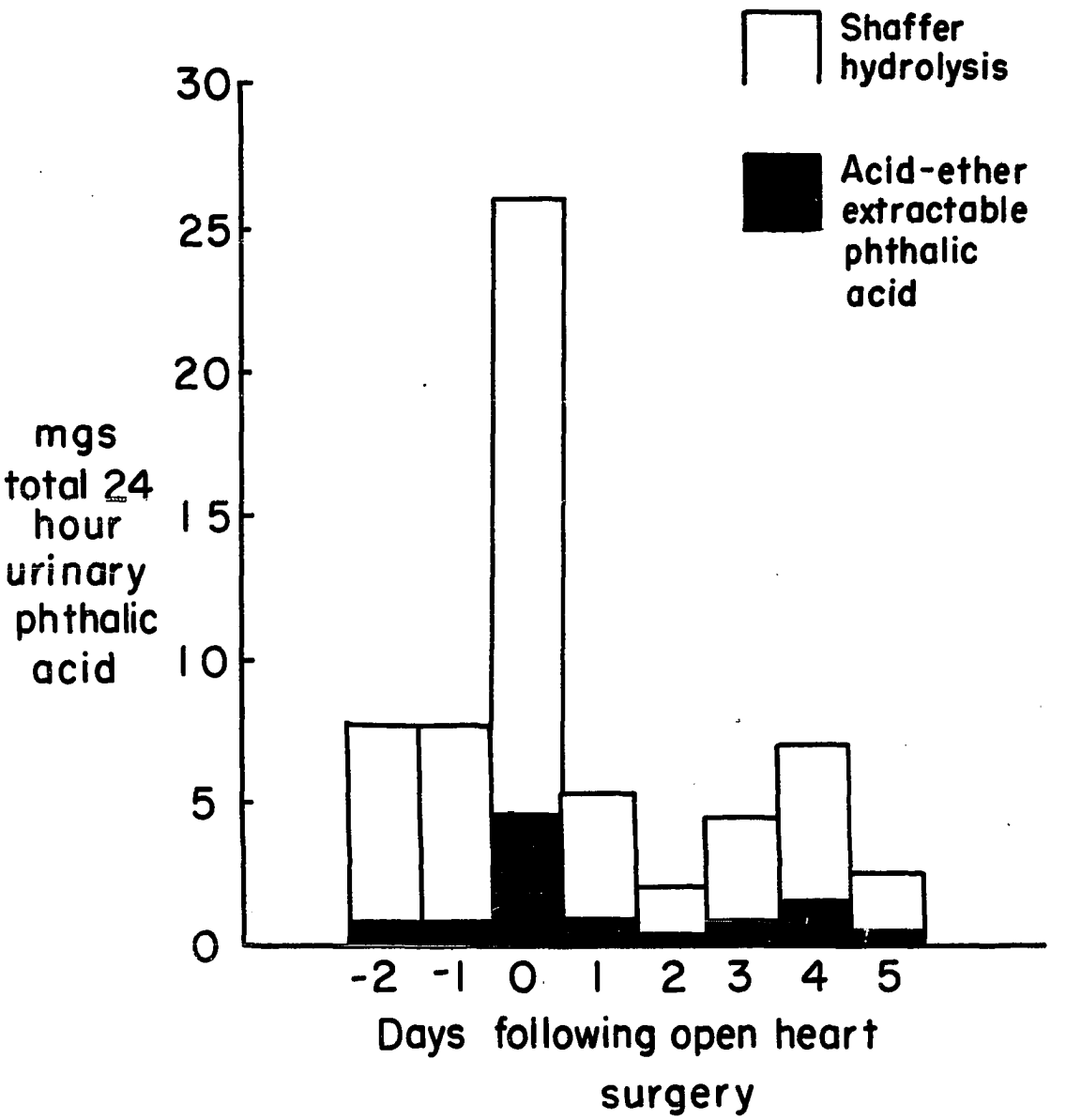
Phthalic acid content of human urine before and after open heart surgery



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increase in urinary "phthalic acid" content when the Shaffer hydrolysis procedure is employed. Therefore, urine from patient RM was hydrolyzed according to the procedure of Shaffer et al and the amount and time course of "phthalic acid" excretion before and after surgery was determined. The results of this analysis are also shown in Figure fifteen (open bars).

It can be calculated that the urinary "phthalic acid" output for this patient on the day of surgery (day zero) is sufficient to account for his having received 43.3 mgs of DEHP. Of this amount, approximately 21 mgs are accounted for by the transfusions that he received, and the remainder must have come from the tubing of the cardiopulmonary bypass system. This result indicates that blood circulating in plastic tubing during open heart surgery is indeed able to extract DEHP. It is also apparent from the figure that this patient was able to metabolize the DEHP rapidly. This rapid metabolism could then explain the earlier findings that not all individuals exposed to plasticized PVC have significant tissue levels of the compound.

A second observation apparent from this figure is that significant urinary "phthalic acid" levels are present before surgery when analysis is done by the Shaffer method. Therefore, it became of interest to examine the urine of normal individuals who had no exposure to PVC medical devices. Early morning urine samples were obtained from three persons and hydrolysis, according to Shaffer's method was performed. This data is shown in table nine. For comparison, the concentration of "phthalic acid" in the urine of patient RM is also shown. It can be seen from the

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Table Nine Urine was collected early in the morning from three normal individuals, and was analyzed by the method of Shaffer et al (1945) as described in methods. In addition, the "phthalic acid" content measured in a similar analysis of the presurgery urine of patient RM is also shown in this table.

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TABLE NINE

HYDROLYSIS BY THE METHOD OF SHAFFER ET AL (1945)

URINARY "PHTHALIC ACID" CONTENT OF NORMAL INDIVIDUALS

SUBJECT	CONCENTRATION mgs PA/100 ml
PATIENT RM	0.48
RJJ	0.36
JK	0.74
JH	1.29

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data that all three normal persons had measurable amounts of urinary "phthalic acid," a result which may indicate some dietary source of this non-biologic acid. However, no conclusion is possible as to the nature of the ester of phthalic acid which lead to the appearance of this metabolite. Phthalates have been reported to be present in food stuffs (Wildbrett et al, 1970; Beroza, et al 1968) and beverages (Cerbulis and Ard, 1967; Kahn, 1969; Kahn et al, 1969). These measurements indicate that the amounts of phthalates consumed per day by normal individuals may be quite significant.

I. Di-2-ethylhexyl Phthalate - Effect on Drug Metabolism

Having established the presence of a foreign compound in the tissues of man, it became of extreme importance to establish the toxicological significance of this observation. The overt hazards associated with phthalate esters are quite minimal (see historical review), and for this reason, the Food and Drug Administration has permitted their use in food packaging materials. However, the inclusion of these FDA approved compounds in plastic medical devices does not guarantee their safety but implies that toxicities directly related to plasticizers have not been described.

Therefore, studies with laboratory animals were performed so that subtle toxicity might be evaluated under more standard conditions than is possible in man. One test of biological derrangement is to measure the rate at which that species is able to metabolize drugs. It is well documented that a number of substances are able to alter the metabolism

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of other chemicals (Conney, 1967; Stitzel et al, 1968). It may be possible that plasticizers can alter the hepatic metabolism of drugs. Therefore, one commonly employed test drug, hexobarbital, was used and the duration of sleep induced by this barbiturate was taken as an indicator of drug metabolism. An alteration in this sleeping time might be indicative of some alteration of this system.

Mice in the 20 gram range were used, and the plasticizer was prepared as an aqueous emulsion in 3% acacia. The solution was injected intraperitoneally at time zero at a dose of 500 mgs/kg. Thirty minutes after the DEHP injection, hexobarbital was administered at a dose of 60 mgs/kg. The duration of barbiturate-induced sleep was taken from the time of injection of hexobarbital to the time that the animals regained their righting reflex. Control animals were given the vehicle instead of the plasticizer at time zero.

These results are shown in figure sixteen. It can be seen from the data that both doses of DEHP (250 and 500 mgs/kg) caused a significant increase in sleeping time while BGBP caused a significant difference only at the higher dose level tested (500 mgs/kg). In the animals treated with either dose of DEHP, a marked difference in appearance was seen when these animals were compared to control or BGBP treated animals. The DEHP groups, thirty minutes after treatment (but before hexobarbital administration), exhibited a reaction which could best be described as characteristic of abdominal irritation, i.e., listlessness, tightening of the abdominal muscles, dragging of the hind limbs, etc. However, these

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Figure 16. Mice used in these exepriments were in the 20 gram range and had been resident in the laboratory for several days during which time they received lab chow and water ad libitum. The mice were randomly asigned to groups, and this figure represents the mean of several experiments.

The animals received the vehicle or the appropriate dose of the plasticizer at time zero. Thirty minutes later, they received hexobarbital at a dose of 60 mgs/kg. The sleeping time was measured from the time of injection to the regain of the righting reflex. The number in parenthesis above each bar represents the total animals in each group.

Hexobarbital was prepared as described in methods. Plasticizer solutions were prepared by sonication of the material in a solution of 3% acacia in saline.

N. S. indicates not significant

* indicates $p < 0.05$

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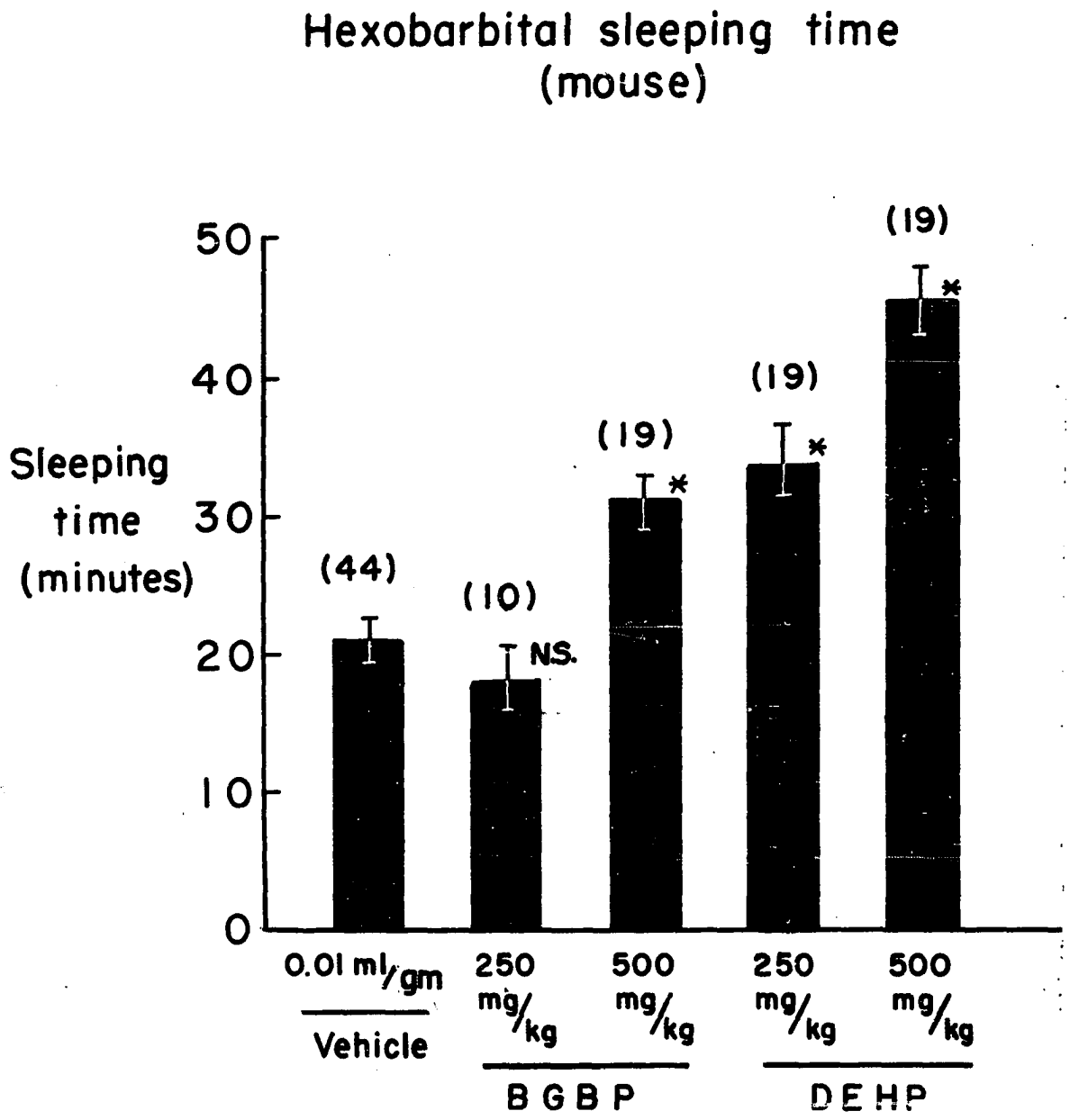

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animals were still able to respond to sensory stimuli.

Repeated daily injections of DEHP at a dose of 250 mgs/kg in mice had a marked effect of body weight. This result is seen in figure seventeen. It can be seen that the rate of weight gain following injections of BGBP or the vehicle were not significantly different from one another. However, DEHP caused the animals to lose approximately 8% of their body weight in the 24 hours after the first injection. With repeated doses, the rate of weight gain was slightly less than controls or BGBP treated mice. It would appear that the mice were able to adapt to most of the effects of this dose of DEHP. However, at the end of the experiment, their body weight was very significantly less than controls or BGBP treated animals.

The effects of DEHP and 2-ethylhexanol on the hexobarbital sleeping time were tested in the rat. This latter compound was tested because the literature indicates that it has anesthetic properties in rats and mice (Hodge, 1943). Thus, deesterification of DEHP might give rise to a substance capable of enhancing the sleeping time due to hexobarbital.

As can be seen in figure eighteen, both DEHP and an equimolar dose of 2-EH caused significant prolongation of hexobarbital sleeping time, but 2-EH had a greater enhancing effect. However, the effects of the two chemicals prior to hexobarbital administration were different. Both rats and mice following DEHP injection (previous experiment) had similar signs of intoxication, in general, signs of abdominal irritation. However, rats given 2-EH were seen to lose their righting reflex prior

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Figure 17. Three groups of ten mice (20 gm range) were weighed for six days. During this time, groups of animals received ip injections of the vehicle (0.01 ml/gm), or BGBP (250 mgs/kg) or DEHP (250 mgs/kg). The plasticizer solutions were prepared daily in a solution of 3% acacia, and the emulsion was formed by sonication.

The animals were weighed and injected at the same time each day, 12 noon. This figure represents the mean cumulative daily weight change of each group, and is based on their initial weight on the first experimental day.

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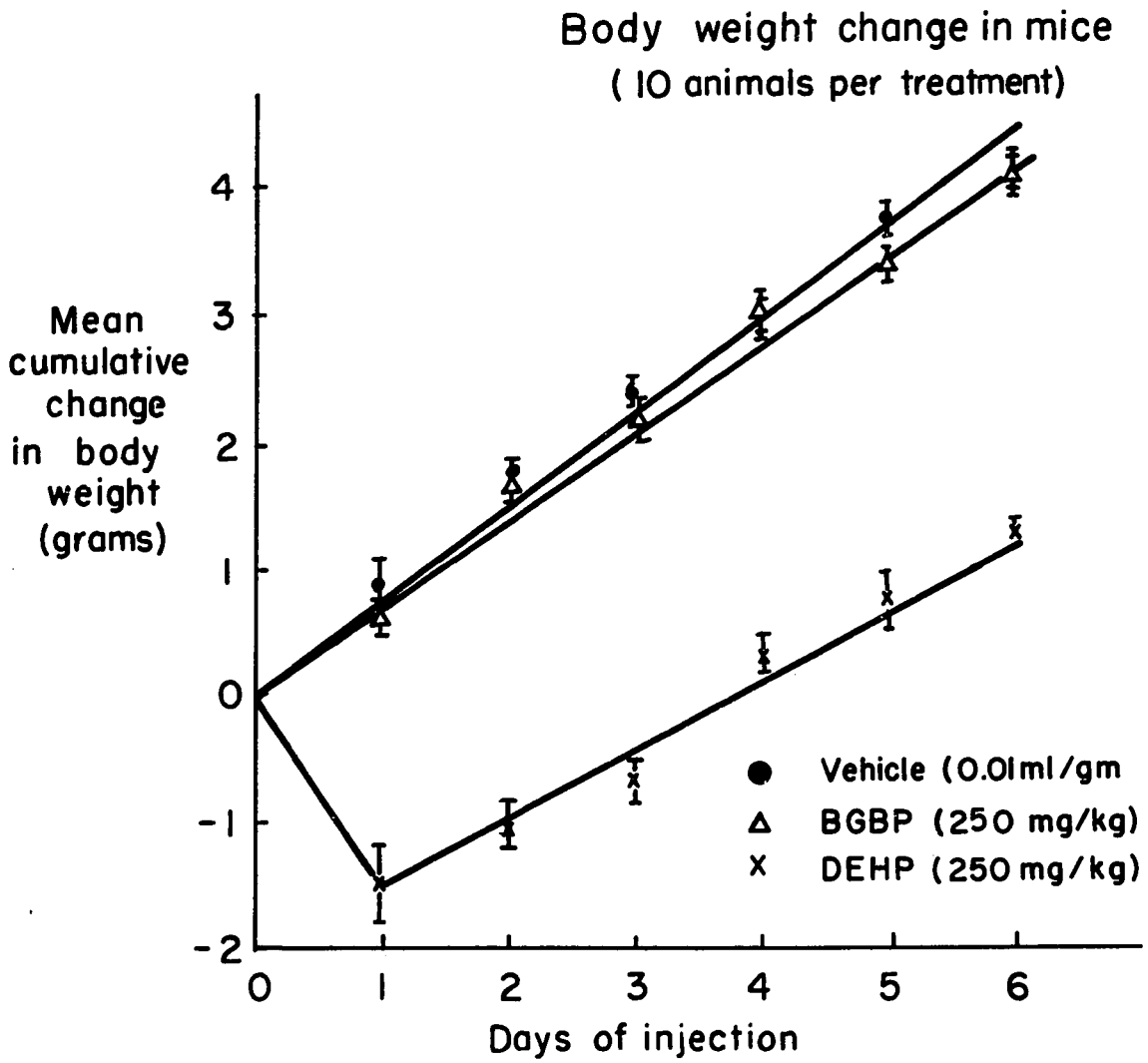

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Figure 18. Rats in the 200 - 250 gram range were used in this experiment. They received the vehicle, DEHP, or 2-EH thirty minutes prior to the injection of hexobarbital. The pretreatment dose was 500 mgs/kg for DEHP or 100 mgs/kg for 2-EH. Hexobarbital was given at a dose of 250 mgs/kg, and was prepared fresh daily as described in methods.

In some experiments, the waking blood level of hexobarbital was determined by the method of Cooper and Brodie (1955) as modified in this laboratory (see methods). Blood samples were drawn from the abdominal vena cava using heparinized syringes. These samples were drawn from etherized animals immediately upon regain of the righting reflex. Samples were analyzed the same day or they were stored frozen overnight.

* Indicates $p < 0.05$

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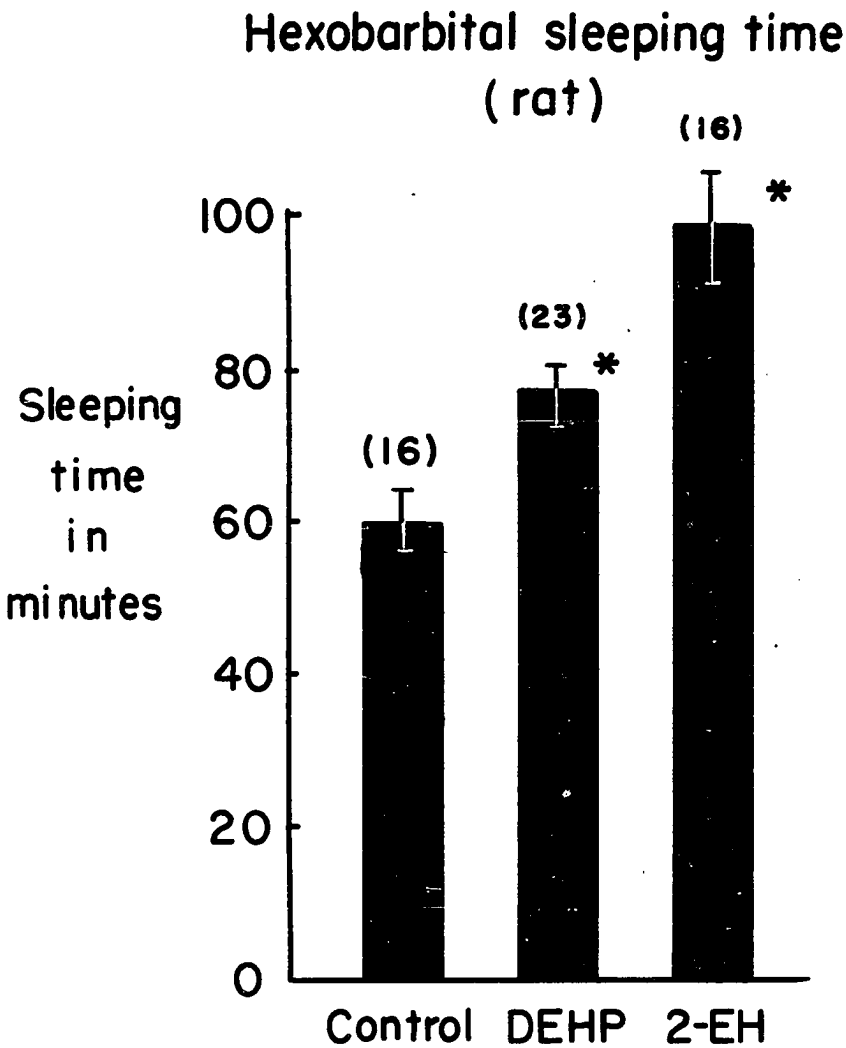

Waking blood levels of hexobarbital μg/ml blood	36.1±3.0 (9)	35.6±2.3 (10)	30.6±2.7 (9)

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to barbiturate administration. Such a loss of righting reflex could be taken to imply a central nervous system depression, and thus, the waking blood levels of hexobarbital might indicate the mode of action of DEHP or 2-EH. These data, also shown in figure eighteen,are the levels of hexobarbital at which animals awakened. It can be seen that none of the treatments causes an increased sensitivity to the drug as evidenced by a lower waking blood level, e. g., lower drug concentration causing sleep. Waking levels of 2-EH while numerically lower than control, were not significantly different from control. Thus, it must be concluded that neither DEHP nor 2-EH increases sleeping time by causing an increased brain sensitivity to the drug, hexobarbital.

Upon laporotomy for the purpose of removing blood samples, it could be seen that, in animals treated with DEHP, large white masses were present on the peritoneal wall and on the surfaces of various organs. A typical example of this is shown in figures ninteen A and B. Histopathological examination of masses found on the spleen and abdominal wall of animals other than the one shown in figures nineteen indicated that the masses consisted "of degenerating neutrophils and some macrophages containing clear vacuoles held together by serofibrinous material . . . The peritoneal surface has focal aggregates of neutrophils and vacuole containing histiocytes within a serofibrinous material containing larger vacuoles." The impression was that "The only changes apparently related to the presence of the plasticizer are focal acute exudative peritonitis suggesting that it has irritating properties" (Montali, 1971.

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Figure 19. Several animals were given an injection of DEHP (500 mgs/kg) as an aqueous emulsion in 3% acacia. Three days after the injection, they were autopsied. The animal shown here is representative of the group.

The position of the white masses was variable from animal to animal. In some cases, they were seen adherent to the omentum, and in other cases they formed on the liver or diaphragm. Control animals injected only with the vehicle did not show any effect.

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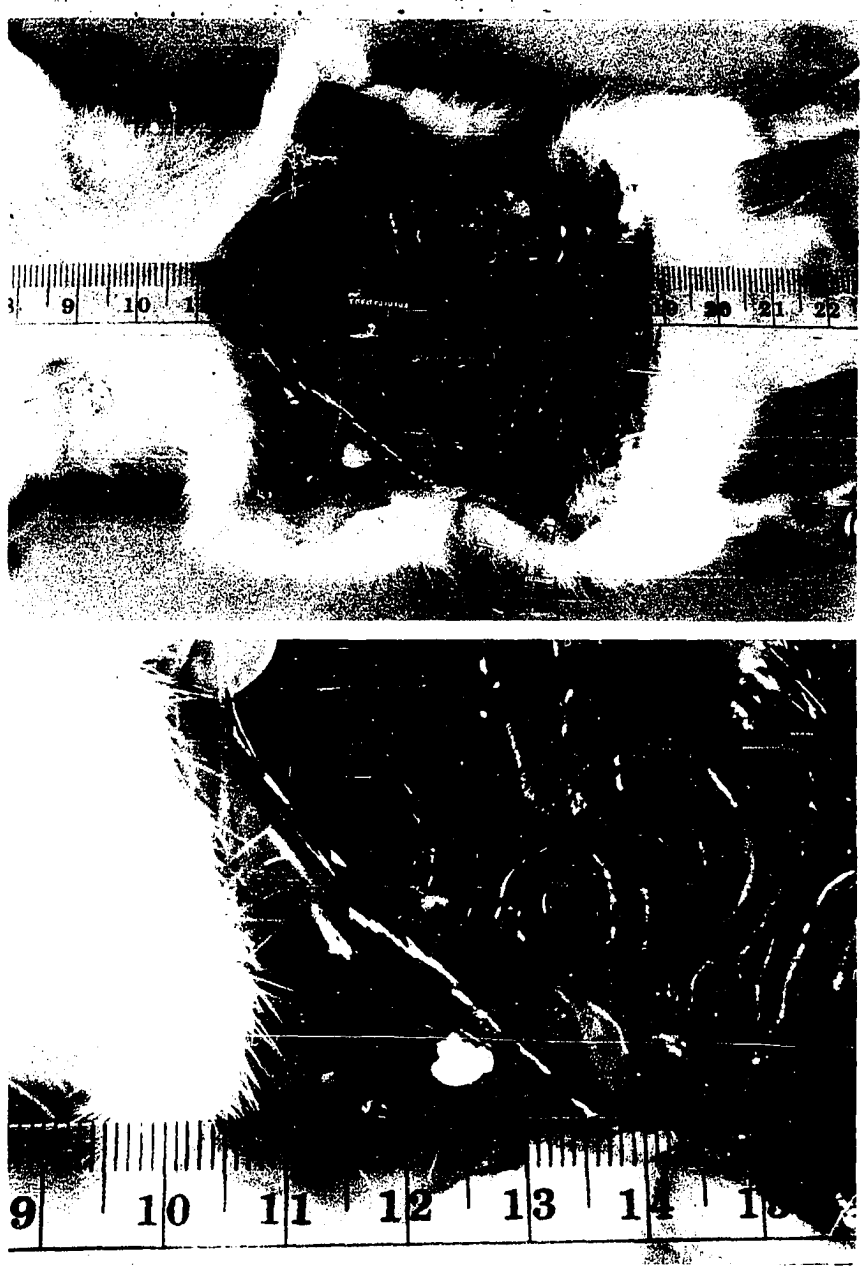
GROSS PATHOLOGY FOLLOWING INTRAPERITONEAL

ADMINISTRATION OF DEHP (RAT)



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The ability of DEHP to prolong hexobarbital sleeping time in vivo without any significant alteration in the waking blood levels of the barbiturate indicates that the effect is related to a decreased rate of disappearance of hexobarbital from the blood. Since a major route for the elimination of a drug from the body is via hepatic metabolism, the effect of DEHP on the rate of hexobarbital metabolism in the isolated, perfused liver was studied. DEHP was added to the perfusate prior to the installation of the liver, and the dose of hexobarbital was given after two hours of perfusion. It can be seen from figure twenty that the rate of disappearance of hexobarbital in this system is not markedly different between control and DEHP treated livers. In fact, as was previously mentioned (section D), 91% of the added DEHP could be recovered from the liver after 4.5 hours of perfusion. Thus it is apparent that DEHP does not cause a marked alteration in the rate of hepatic hexobarbital metabolism, and thus, such an effect cannot be used to explain the marked prolongation of hexobarbital sleeping time after DEHP administration.

DEHP and BGBP were tested for their effect on the duration of action of a spinal paralytic, zoxazolamine. This drug, unlike hexobarbital, does not have a central nervous system effect but rather inhibits multisynaptic pathways in the spinal cord (Kamijo and Koelle, 1955). As can be seen from the data shown in figure twenty-one, neither BGBP nor DEHP altered the paralysis time nor the blood level at which the animals regained their righting reflex ($P>0.05$ from control). The results of this experiment may be used to argue that the prolongation of

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Figure 20. Isolated perfused liver experiments were done as described in methods. DEHP in perfusion plasma was prepared by sonication so that the initial level (time zero) would be 70 µg/ml. The system was allowed to equilibrate for two hours before the hexobarbital was administered.

Hexobarbital, approximately 10 mgs, was added to the main perfusate reservoir. Blood samples were taken at 5, 15, and 30 minutes from the blood in this reservoir. Hexobarbital analysis was performed as described in methods.

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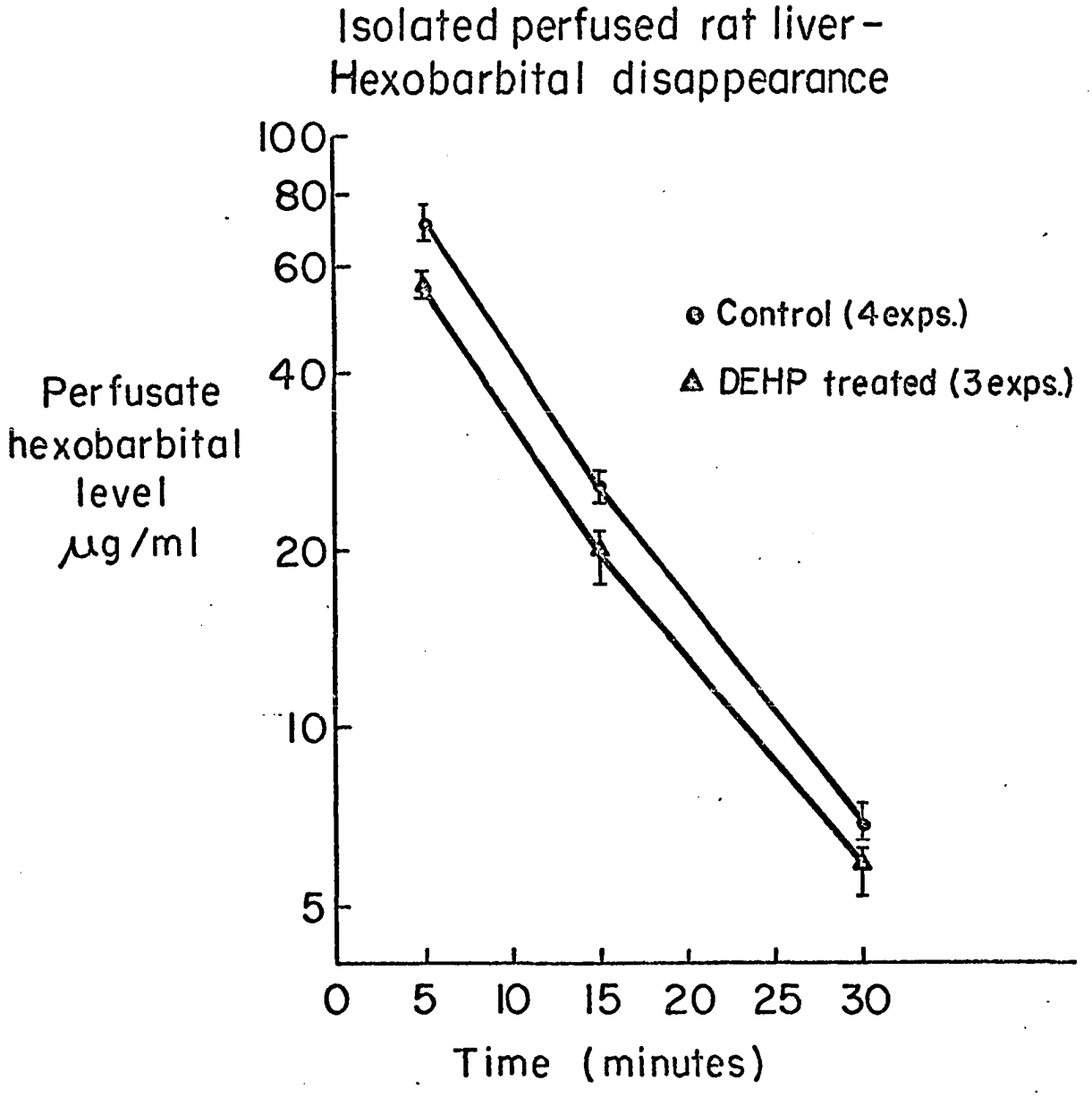

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Figure 21. Rats in the 200 - 225 gram range were used in these experiments as described in methods for sleeping time determinations. They were given the plasticizer (500 mg/kg) or the vehicle (1ml/100gms) thirty minutes prior to zoxazolamine (75 mg/kg).

The paralysis time was taken from the time of injection to the regain of the right reflex. Blood samples from the abdominal vena cava were analyzed for zoxazolamine as described in methods.

N.S. indicates not significant ($p > 0.05$)

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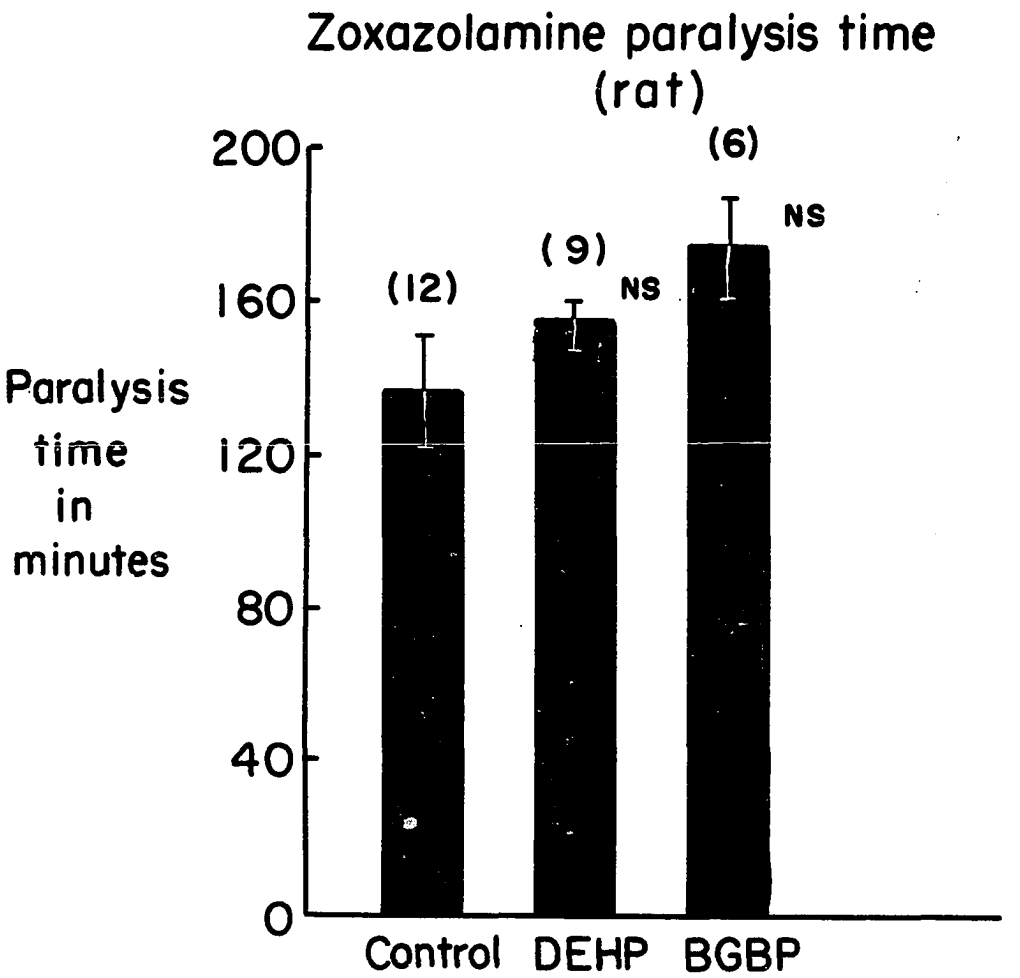

Waking blood levels of zoxazolamine μg/ml blood	Control	DEHP	BGBP
	47.1 ± 1.4	45.5 ± 2.0	49.3 ± 1.3

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hexobarbital sleeping time seen after DEHP administration in the intact animal is not due to an altered uptake of the drug from the peritoneal cavity since zoxazolamine is presumably absorbed in a similar manner to hexobarbital. At present the reasons for prolongation of hexobarbital sleeping after DEHP administration are not apparent.

J. Di-2-Ethylhexyl Phthalate - Behavioral Studies

As was reported in an earlier section, DEHP was seen to cause a visually observable depression in gross behavioral activity in the rat and mouse as well as marked changes in body weight in the mouse. Thus, behavioral effects were evaluated in a more systematic, quantitative manner in the rat. Behavior was evaluated in two ways: 1. lever pressing in response to a pleasurable hypothalamic shock, and 2. spontaneous running in an activity wheel. In the second experiment (activity wheel), daily weight gain was recorded.

The results of the hypothalmic electrode study are seen in figure twenty-two. Animals were prepared as described in methods, and the lever pressing rates prior to treatment with DEHP were determined. The average daily standard error of the assay is shown in the figure as the cross hatched area and is 7% of the control rate. When DEHP was administered intraperitoneally two hours prior to the daily one hour test period, it can be seen that a very profound decrease in the response rate occurred. This highly significant result ($p < 0.01$) indicates that the animals did not perform the learned response after DEHP treatment.

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Figure 22. Rats in the 350 gram range (Long Evans hooded strain) were prepared with hypothalamic electrodes. They were trained to press a lever for a reinforcing stimulus, and the mean percent variability for the assay is shown as the cross hatched area in this figure.

DEHP (500 mg/kg ip) was given two hours before the animals were tested, and the value shown in this figure is the average of the animals performance after this dose. Following a period of two weeks, during which time the animals returned to control rate they were treated with the vehicle (3% acacia in saline, 1 ml/100 gms) and their rate was again determined.

* indicates $p<0.01$

N.S. indicates not significant ($p>0.05$)

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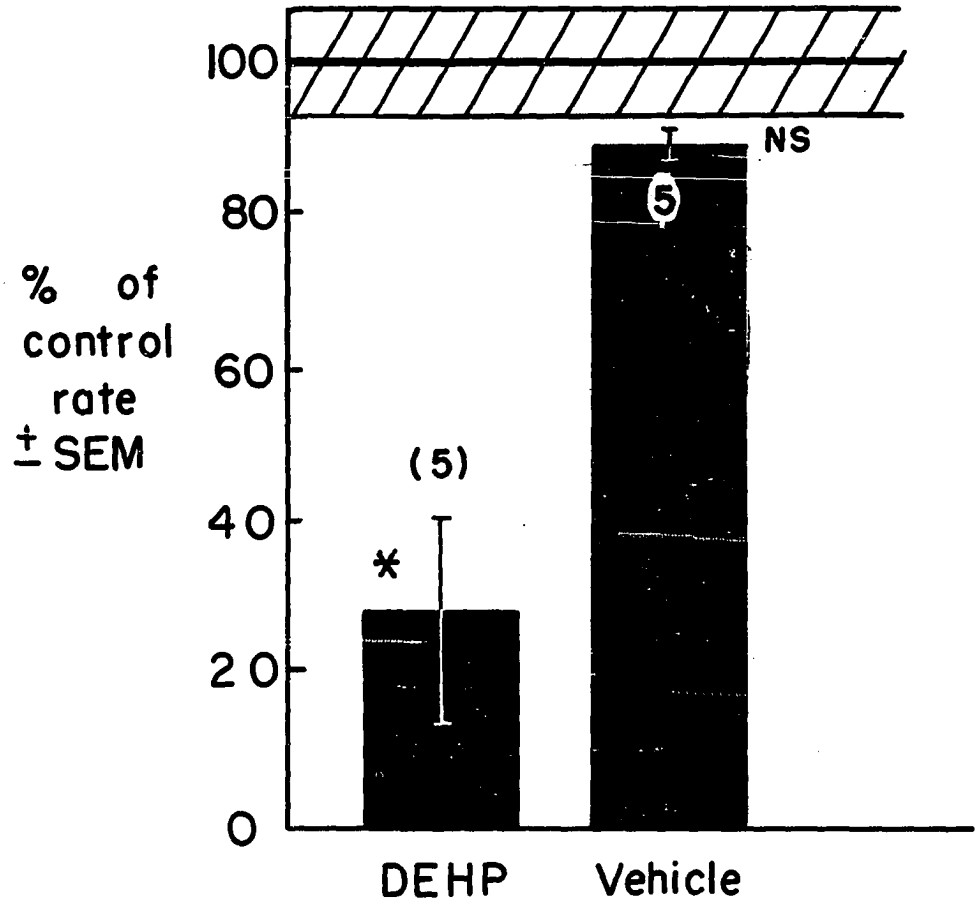
Hypothalamic self stimulation rate during a one hour trial period.



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After a recovery period, the same animals were treated with the vehicle, and it can be seen from the data that this did not cause a marked alteration in behavior.

Similar results were obtained with other animals allowed free access to activity wheels. This data is shown in figure twenty-three. The cross hatched area at the top of the figure indicates the mean variability in the animals' day to day activity. When DEHP was given at 12 noon, the following total 24 hour running activity was severely depressed. After a recovery period, the vehicle was injected . While this treatment did have an effect on activity, the decrease due to DEHP was still highly significant ($p < 0.01$) when compared to the vehicle treated group.

During this experiment, it was seen that animals had a very marked decrease in body weight during the twenty-four hours which followed DEHP treatment. The changes that were observed in this experiment are shown in table ten. It can be seen that the single 500 mg/kg injection of DEHP causes a very significant ($p < 0.01$) loss of body weight.

A further experiment was conducted to determine the effect of the plasticizer, DEHP, on the amounts of food and water consumed during 24 hours. Thus, losses in body weight after treatment with DEHP could be explained on the basis of a decreased food consumption or perhaps a decreased food utilization. Therefore, two animals were placed in special chambers which required that levers be pressed for both food and water. Special counting devices recorded the hourly total of each that were consumed during the experimental period. The results of this experiment are shown in figure twenty-four. It can be seen that the

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Figure 23. Male Carworth Farms rats in the 250 - 300 gram range were housed in activity wheel cages, and were given food and water ad libitum. Each animal's running activity was measured at 12 noon each day, and injections of DEHP were given at this time. The vehicle was given to the same animals after a period of time (two weeks) was allowed for recovery. The total activity for the subsequent 24 hour period was determined.

The value shown in the cross hatched area is the mean standard error for all the rats. The data was analyzed as paired groups (each animal acted as his own control).

* indicates $p<0.01$ - significantly different from vehicle treatment

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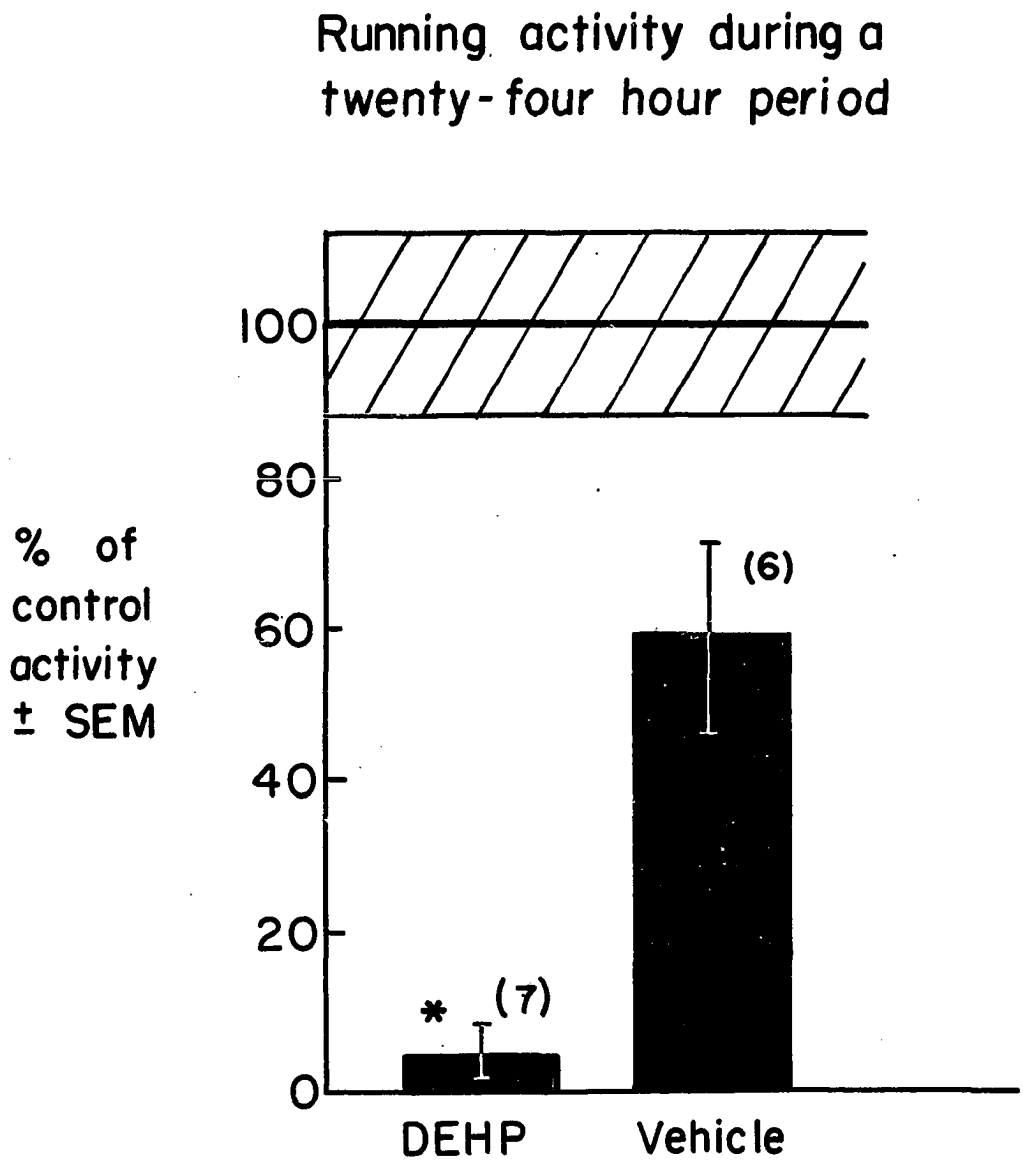

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Table Ten. The data in this table represents the daily changes in body weight for animals housed in activity wheel cages. All weights were recorded at 12 noon and injections of DEHP (500 mg/kg) or the vehicle (1 ml/100 gm) were given at this time. The data in this table corresponds to the data shown in figure 23.

* indicates $p<0.01$ - significantly different from pretreatment control or from vehicle treatment

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TABLE TEN

BODY WEIGHT CHANGES IN RATS ALLOWED FREE ACCESS TO ACTIVITY WHEELS

		BODY WEIGHT CHANGE GRAMS/RAT/DAY
PRETREATMENT CONTROL	(7)	+ 1.9 ± 0.5
DI-2-ETHYLHEXYL PHTHALATE	(7)	- 24 ± 1.7 *
VEHICLE TREATMENT	(6)	+ 2.7 ± 1.1

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Figure 24. Two animals, male albino CFN rats were placed in special cages that permitted measurement of their food and water consumption. They were allowed several days to acclimate to their surroundings before measurements were made. The vehicle or DEHP was given at 12 noon, and the 24 hour average of hourly food consumption was measured.

The average loss in body weight on day 6 (24 hours following DEHP) was 24 grams, while no change in body weight occurred during the 24 hours following the vehicle injection (day 5).

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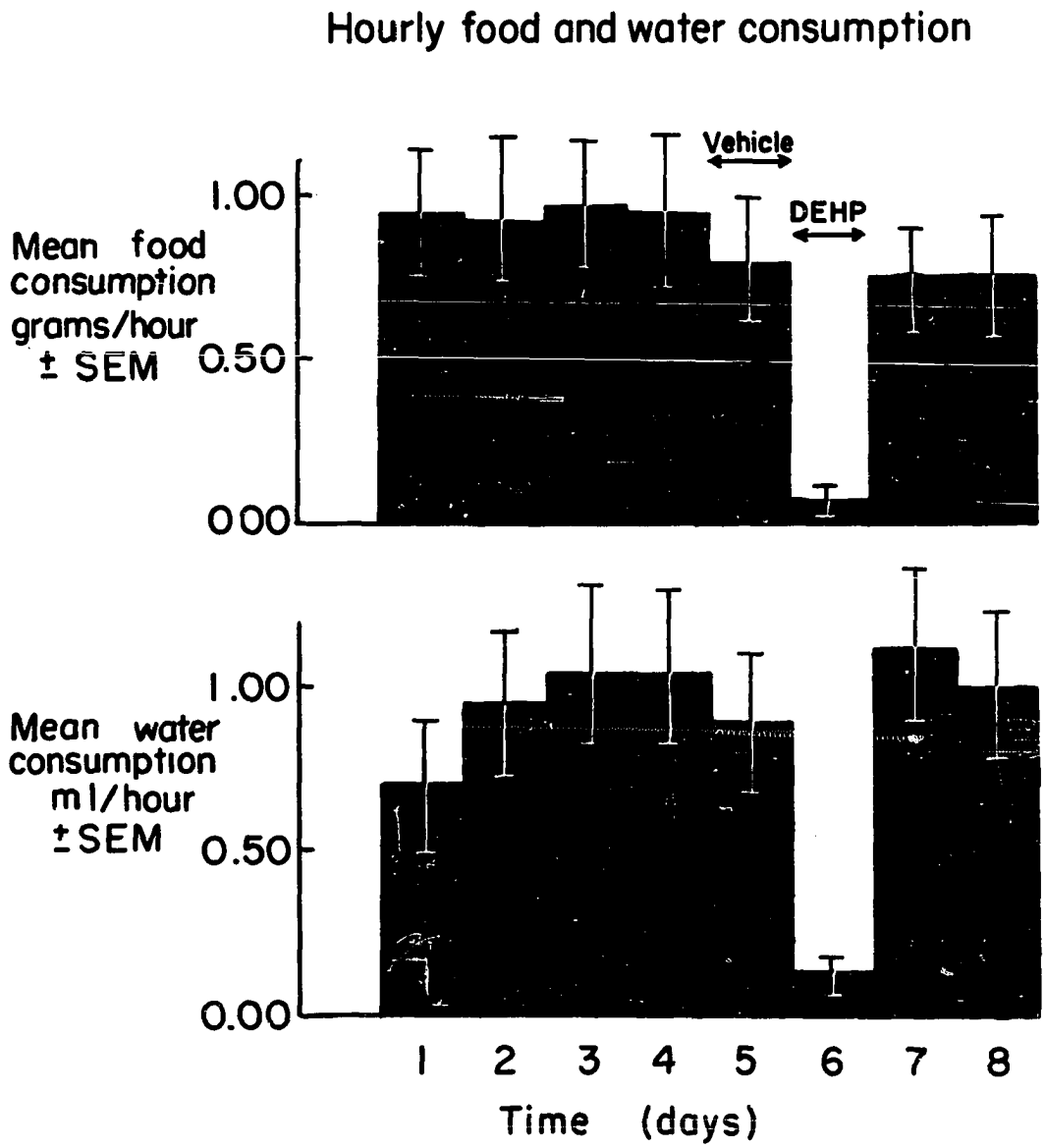

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vehicle, given 24 hours before the plasticizer, had little effect on the hourly rate of food or water consumed. However, the plasticizer caused a very profound decrease in the hourly food and water consumption. This effect lasted about 24 hours and animals returned to normal in the following 24 hour period. Thus, it can be seen from this data that the pronounced loss in body weight after DEHP treatment is a result of a decreased intake of both food and water.

It became of considerable interest to determine whether a similar effect on body weight would occur after intravenous administration of the plasticizer, since this route might approximate human exposure. It can be seen in figure twenty-five that a dose of DEHP (500 mg/kg) given by an iv route causes a highly significant weight loss while animals given only the vehicle show an increase in weight. The net effect of this dose of the plasticizer is a 2% fall in body weight over the 24 hours after injection of the plasticizer.

It can be concluded from these studies that this dose of DEHP (500 mg/kg) given intraperitoneally causes a very marked effect on behavior as reflected by a decreased food and water consumption which results in a large decrease in body weight. A similar dose of DEHP given intravenously, also causes a loss in body weight. While this latter effect is highly significant ($p<0.01$), it is numerically less than the weight loss seen after intraperitoneal administration.

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Figure 25. Male albino rats, 200 - 225 gram range, were given iv injections of DEHP (500 mg/kg) or the vehicle (3% acacia, 1 ml/100 gms). Their change in body weight over the subsequent 24 hours was determined.

* indicates $p<0.05$

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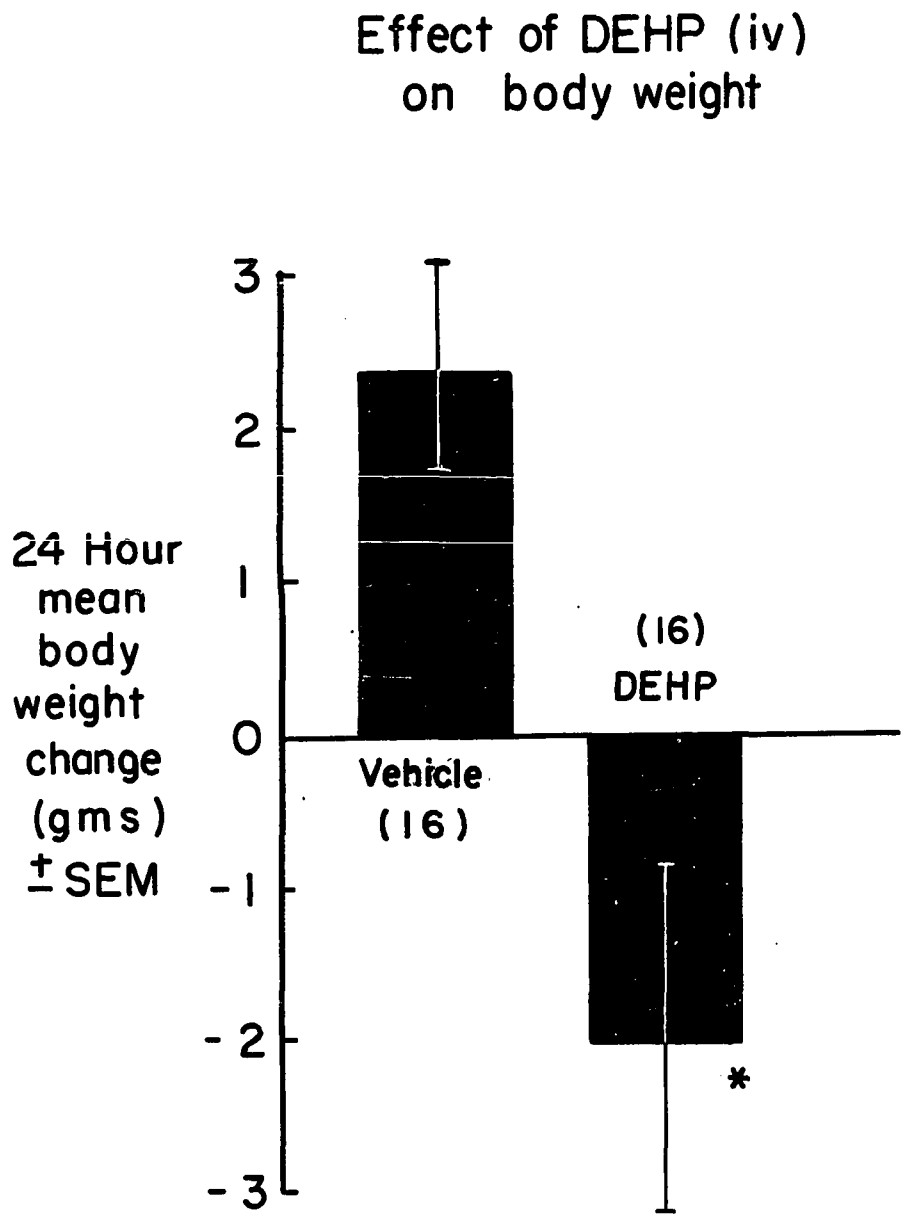

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K. Di-2-ethylhexyl Phthalate - Effect on Reticuloendothelial Function

It has been shown (Section E), that DEHP is accumulated in the lung, liver and spleen, tissues usually associated with reticulo-endothelial function. Such tissues are involved in the removal of particles such as bacteria (Horn et al, 1969) or emulsified lipids (Edgren, 1960) from the circulation. Therefore, experiments were performed to determine if DEHP as an emulsion could either inhibit or enhance the rate of RE clearance of a test particle suspension, in this case, particles of colloidal carbon. Two experiments, separated by several months, were performed. Injections of DEHP were given in the following way: 1. a single iv dose of the plasticizer at a level of 500 mg/kg, and 2. 125 mg/kg given on alternate days for a total of four injections. Carbon clearance was measured twenty four hours after the last dose of DEHP. Figure twenty-six A and B illustrates the result of these experiments.

It can be seen that the dose of carbon is cleared in logarithmic fashion in the controls animals of each experiment. The two experimental controls differ slightly in the half time of carbon clearance (5 minutes in experiment one, and 4 minutes in experiment two). It is presently not known why the two control rates differed between these two experiments. However, the several month interval between experiments may have resulted in a subtle alteration in RE function of the two groups of animals.

It can be seen from the data presented in the figures that the effect of a single injection of DEHP is such that the rate of carbon clearance is depressed after 24 hours. The magnitude of the depression differs

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Figure 26 A and B. Male albino rats were given intravenous amounts of DEHP or the vehicle. Twenty-four hours after the last dose, carbon clearance was assessed as described in methods.

Animals were allowed free access to food and water after the pretreatment. In cases where several injections of the plasticizer were given, animals were also given injections of the acacia vehicle. Single injections or multiple injections of the vehicle did not alter carbon clearance rates. Therefore, all animals who received the vehicle are pooled within the control group.

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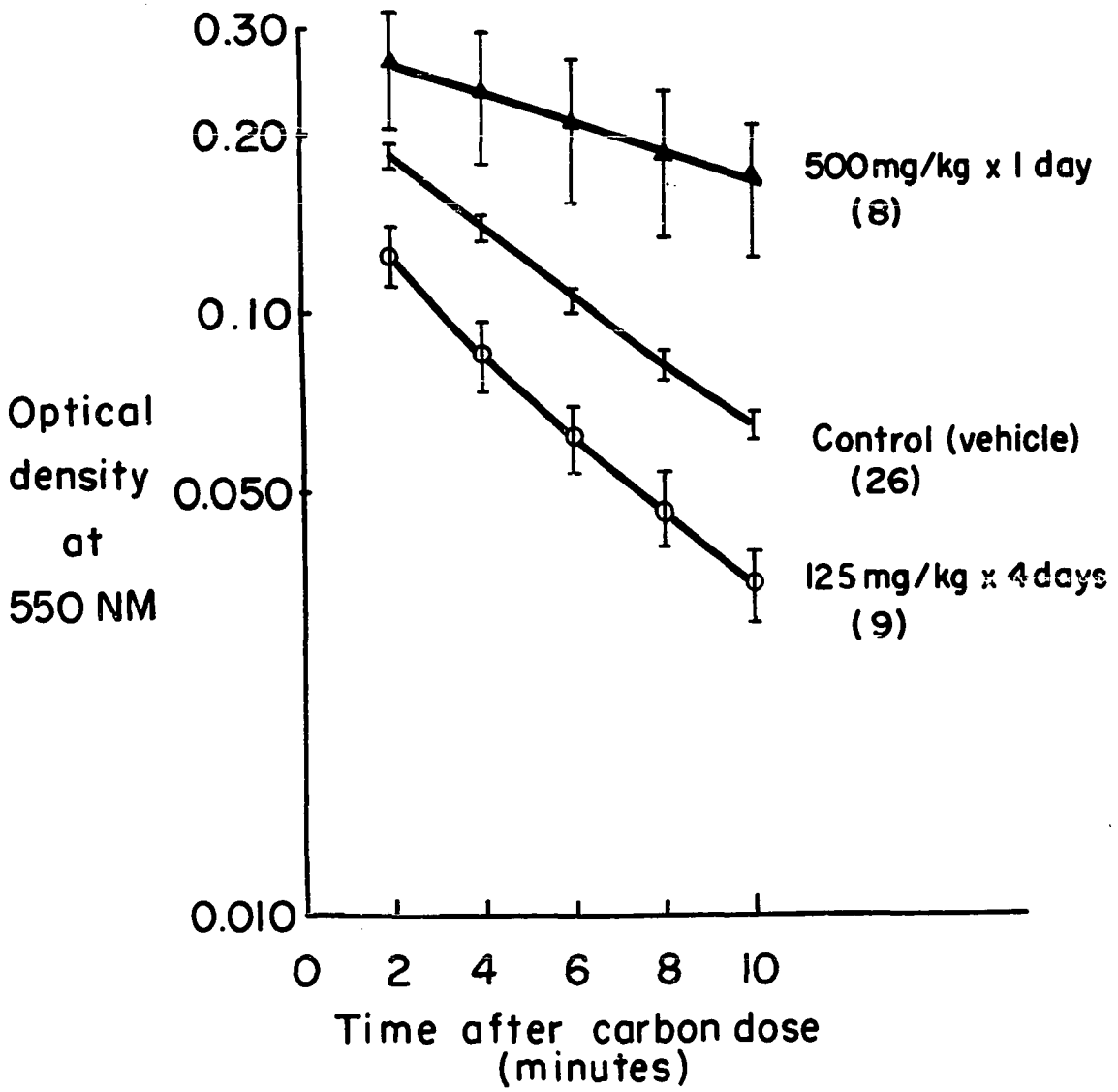
A



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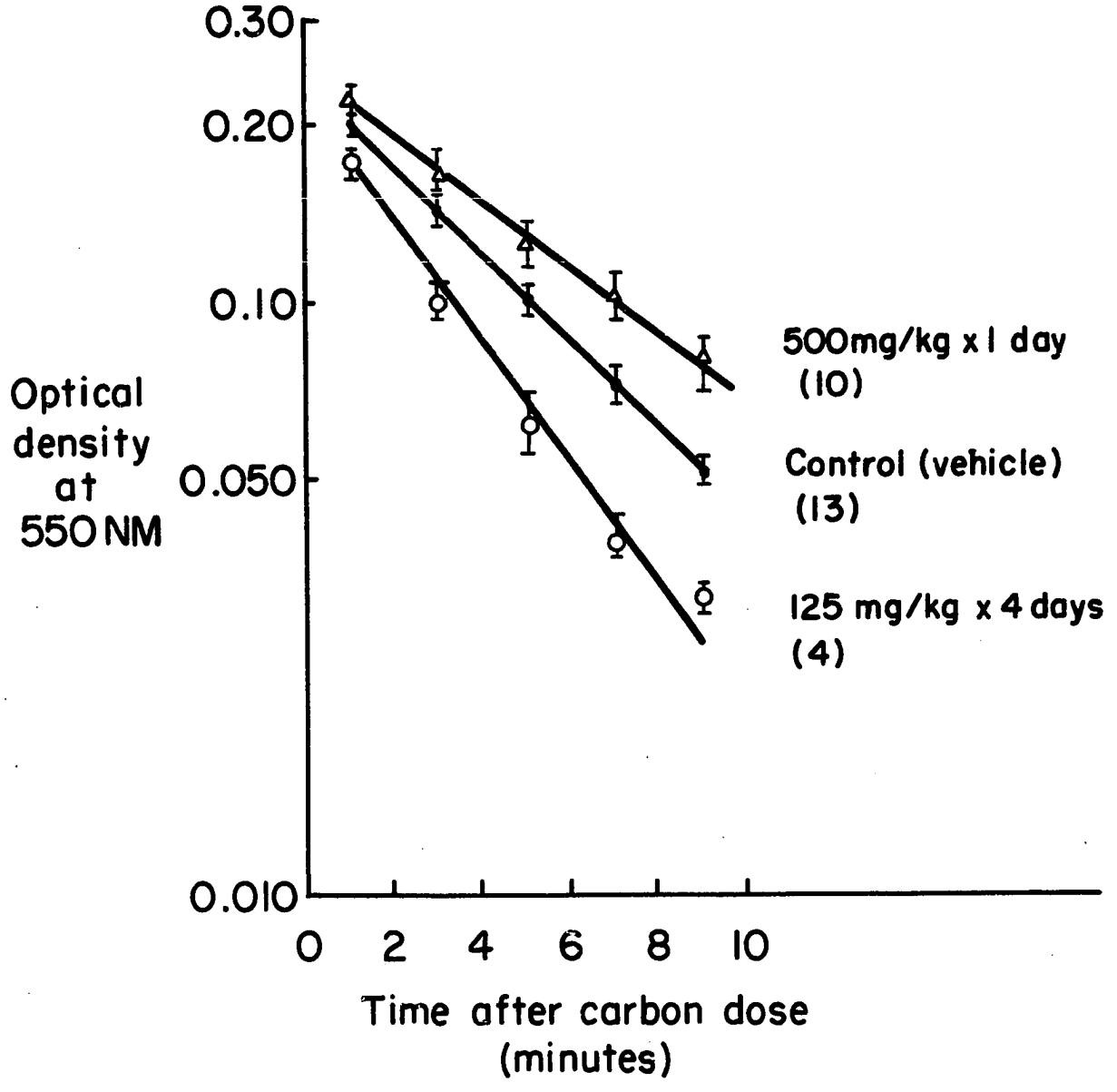
B

Carbon clearance in rats –
Effects of DEHP iv exp#2



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in the two experiments; but in both cases, the acute effect of the plasticizer is the same, one of depression of reticulo-endothelial function.

In the case of the experiments where four doses of DEHP were given, it can be seen that carbon dissappears from the blood stream more rapidly. In the first experiment, the rate between control and treated animals does not differ. However, the concentration of carbon is reduced at the first, and all successive times, relative to control. In the second experiment, the concentration of carbon in the blood falls more rapidly in the DEHP treated animals than in control. It is not apparent why differences exist between the two groups and their response to multiple DEHP injections. However, it is apparent that the intravenous administration of the plasticizer, DEHP, is able to alter markedly the rate at which particles such as carbon are removed from the blood stream by reticulo-endothelial tissues.

L. Di-2-ethylhexyl Phthalate - Effects on Blood Micro-aggregate Formation

Another aspect of the problem of DEHP contamination is not only the effect that this material has directly on the intact animal but also the effect of the compound on stored blood that is subsequently to be used for transfusions. It has been shown in an earlier section that plastic-stored blood, both dog and human, progressively accumulates DEHP. In addition, it will be shown by the author and it has been shown by McNamara, et al (1970) that human blood, following periods of storage in plastic containers, contains micro-embolic aggregates. Swank (1963) has shown that these

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aggregates disappear from the patient's circulation after the blood is transfused. Further, the aggregates have been identified by Swank (1963) as clumps of platelets and leucocytes, and present efforts have been directed toward the efficient filtration of blood so that these aggregates are removed. A recent report by Mittermayer et al (1970) has suggested that the phenomenon of shock lung (disseminated intravascular coagulation) is a direct consequence of micro-emboli which have lodged in the terminal segments of the pulmonary vascular bed. McNamara et al (1970) have shown that the development of shock lung in combat casualties is highly correlated with multiple transfusion of aged, plastic-stored blood. Therefore, it became pertinent to investigate the relationship between the plasticizer accumulated in stored blood and the formation of micro-aggregates.

A direct measure of the micro-aggregate formation is available by measurement of screen filtration pressure, a procedure developed by Swank (1961). In this test system, blood is pumped at constant flow through a screen of defined cross sectional area having pores of 20 microns diameter. The pressure that develops across the screen is a measure of the occlusion of the pores and the adhesiveness that the occluding particles have for one another.

In this study, canine blood was used as the model and was stored in the following types of plastic containers: 1. commercial PVC blood storage bags (Fenwal JA-2C blood pack, Baxter Laboratories, Morton Grove, Ill.) with anti-coagulant citrate-dextrose solution (ACD-NIH formula A), 2. Fenwal PVC bags specially prepared with an inner surface

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of heparin bonded to graphite (PVC-GBH) containing fresh ACD, and 3, experimental blood bags of Hemosil 103 copolymer (Medical Engineering Corp, Racine, Wisconsin) containing ACD as the anti-coagulant. These latter bags (HS 103) served as the non-plasticized controls.

The results of this experiment, expressed as screen filtration pressure as a function of days of storage in the various blood bags, is shown in figure twenty-seven. It can be seen that PVC stored blood shows an increased SFP with time and the slope of the linear regression equation is 8.96 ± 2.02 mm Hg per day. The blood from PVC-GBH units increased in SFP more slowly than the PVC units and had a significantly lower regression slope (3.38 ± 0.41 mm Hg per day). Both of these rates of increase are significantly greater than zero and are different from one another.

The blood stored in the Hemosil bag does not increase in SFP very rapidly with time; in fact, statistical analysis of the line of best fit between days five and twenty indicates that the slope is not significantly different from zero. The apparent increase in SFP between days zero and five cannot be appropriately evaluated at this time since the blood stored in the Hemosil bags was not assayed at time zero, i.e., prior to storage. The possibility remains that these units of blood had a finite SFP prior to storage. The main point to be made is that there is little further increase in SFP with storage.

Measurements of the amounts of plasticizer in blood stored in the two PVC containers indicated no difference in the rate of accumulation of DEHP; this result can be seen in figure twenty-eight. Since blood

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Figure 27. Seven units of dog blood were collected in plastic blood storage bags. Three units were drawn into standard blood bags (Fenwal JA-2C blood pack, Baxter Laboratories, Morton Grove, Ill.) containing 67.5 ml of ACD as the anti-coagulant. Two units were drawn into specially prepared PVC blood bags which had an inner surface of heparin bonded to graphite, also with ACD as the anti-coagulant. Two additional units were drawn in special plastic bags formulated of Hemosil 103 (Medical Engineering Corp., Racine, Wisconsin). ACD was the anti-coagulant.

SFP measurements were done as described in methods at the laboratory of Dr. Tom Solis, Walter Reed Army Hospital. Each unit was sampled serially at the times indicated except a single PVC unit which was measured at less than one day of storage. This unit was tested only one time and then discarded.

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Screen filtration pressure of plastic bag stored blood versus days of storage

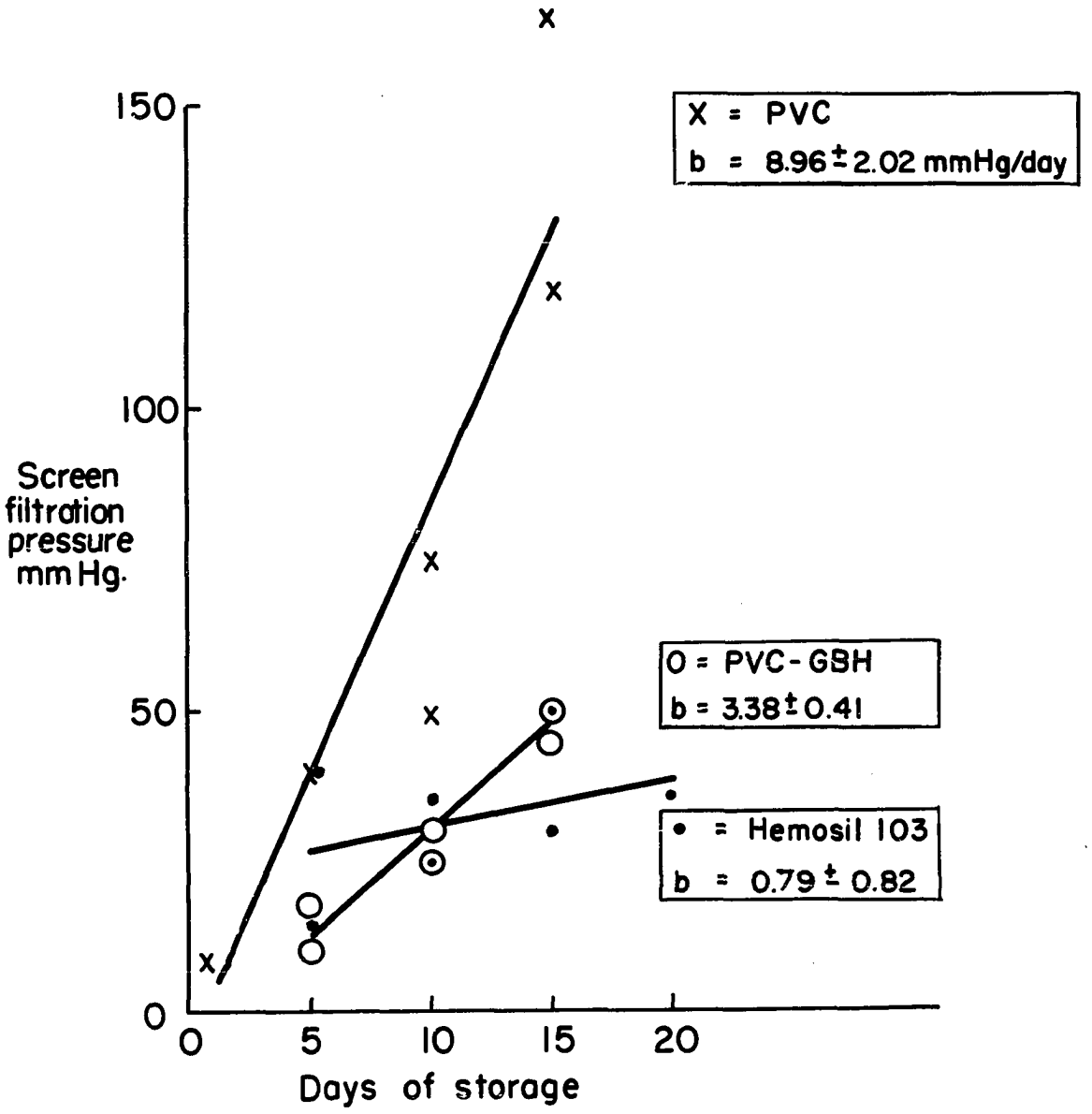

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Figure 28. Units of dog blood, stored in PVC plastic bags with or without heparin coating, were analyzed for their DEHP content as described in methods. The units were sampled repeatedly except for a single unit analyzed at less than one day of storage. This unit was discarded after the first day.

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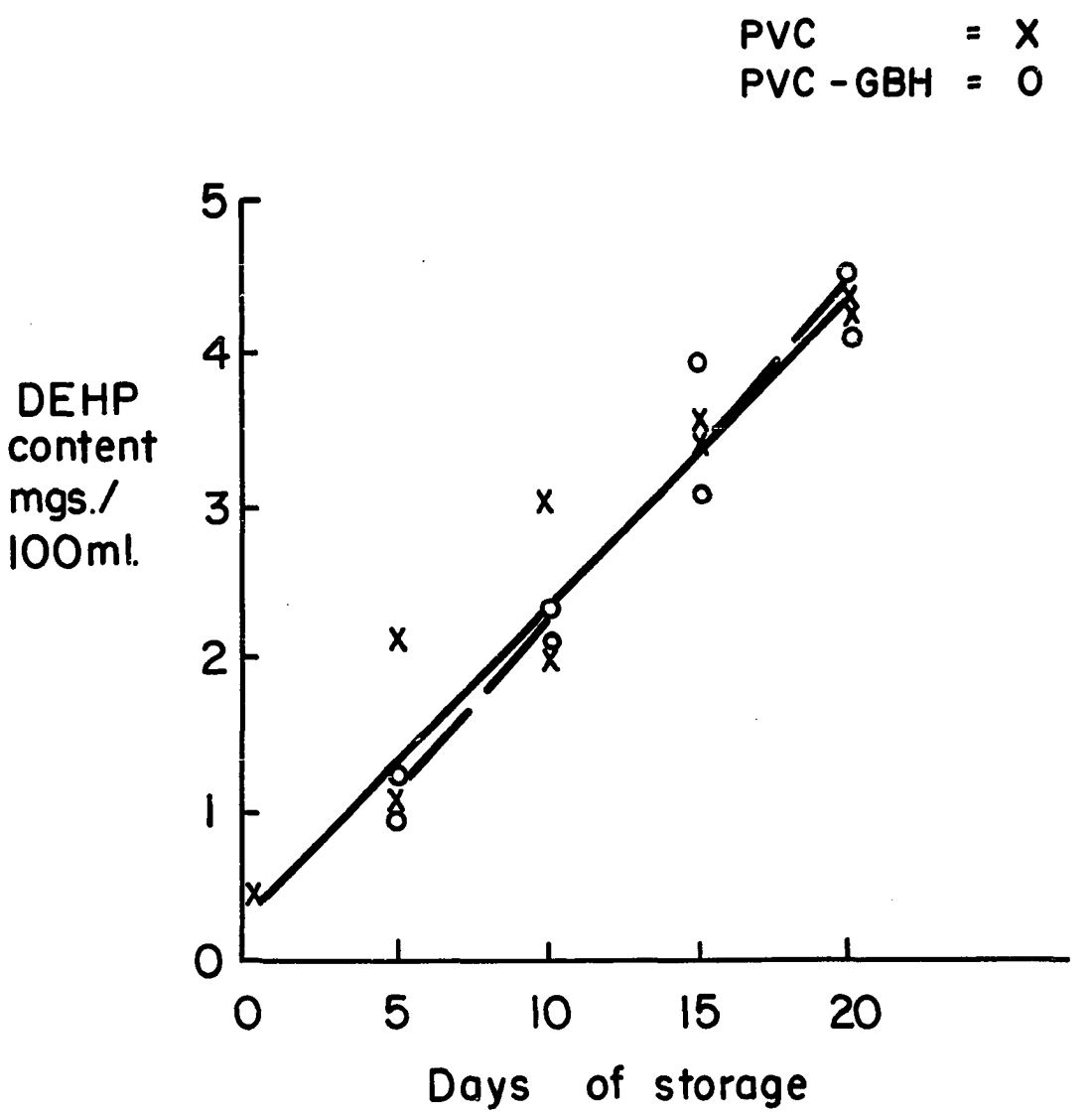
DEHP content of whole blood stored in PVC plastic blood bags



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stored in the two types of PVC containers exhibit markedly different SFP values in the face of equivalent concentrations of DEHP, the plasticizer alone cannot account entirely for the rate of SFP increase in the PVC plastic stored blood.

In figure twenty-nine the correlations of DEHP content with SFP for the two PVC bags are shown. The correlations are highly significant and further, indicate that, for a given level of DEHP, there is a correspondingly larger increase in SFP for the contents of the PVC bag than for that in the PVC-GBH bag. That is, the SFP of the blood in the PVC bag is more sensitive to any given level of DEHP. These results can be interpreted to indicate that the ultimate development of an elevated SFP may depend on at least two factors, the DEHP content and the presence of a certain type of bag surface. When the bag surface is altered, as in the PVC-GBH case, the full effect of the DEHP is not evident. Thus, the data indicates a relationship between DEHP content and screen filtration pressure of stored blood. However, it should be emphasized that a correlation does not imply cause and effect relationship. The increase in SFP could result from an, as yet, unknown process which also happens to be correlated to the DEHP content of stored blood.

This process of aggregation may be related to the life span of blood platelets, and thus, given sufficient time, even the container of Hemosil might form platelet aggregates. However, this container contains no plasticizer, and within the normal storage time of blood (21 days), blood stored in this container did not significantly change in SFP from day five to day twenty. Thus, this material may allow better

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Figure 29. Screen filtration pressures and DEHP content were determined as described in methods. The data is plotted as the line of best fit, and the correlation coefficient was determined using Pearson's rank correlation r value (zero order). The slopes of the lines indicated that they are significantly different from one another.

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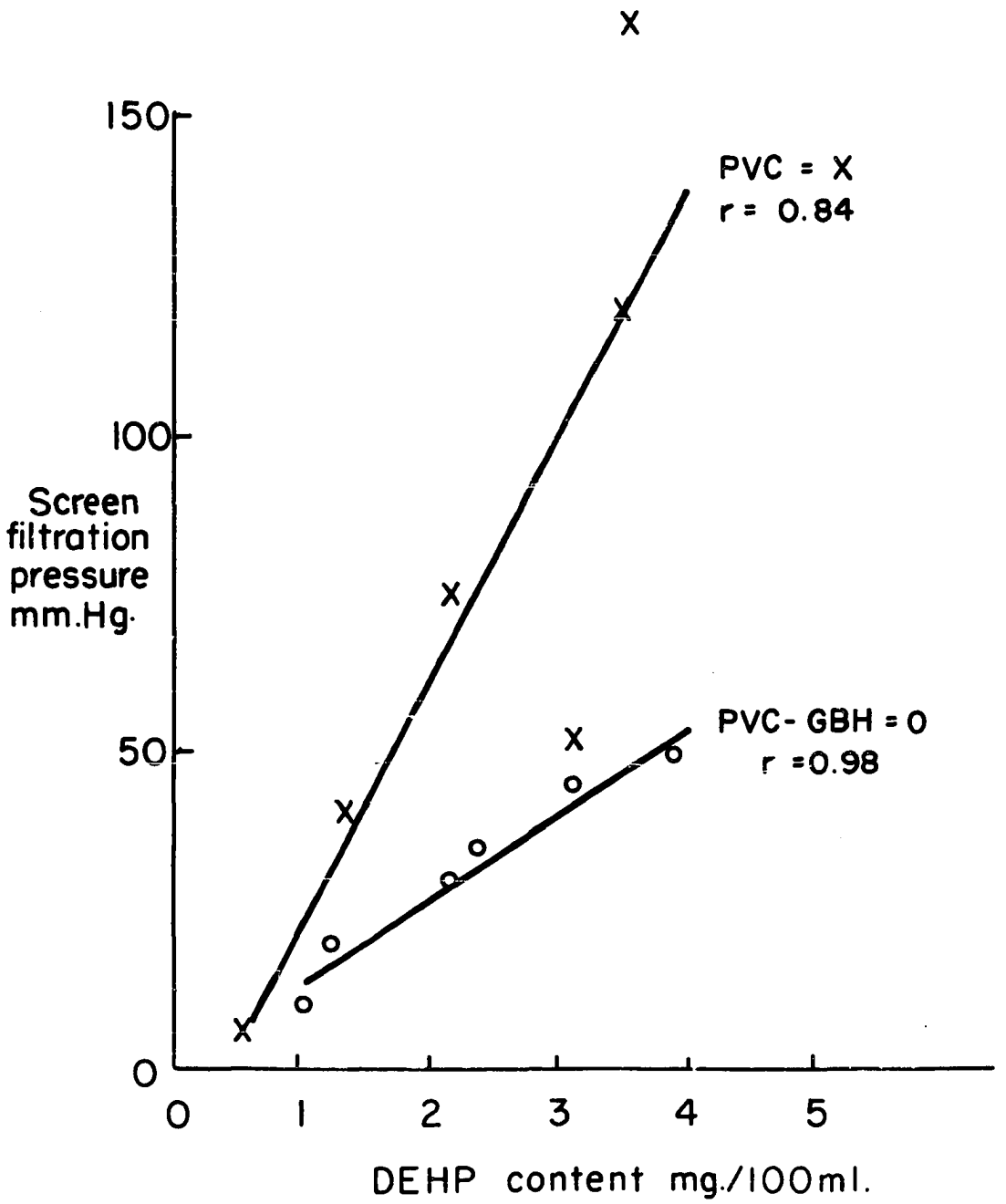
Screen filtration pressure versus DEHP content of PVC stored blood



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preservation of platelets, and could represent a significant improvement in the storage of whole blood.

M. Di-2-ethylhexyl Phthalate - Isolation of Plasticizer from Filtered Blood Clots, and Units of Two day Stored Platelet Concentrates

When units of blood are transfused, relatively coarse filters are placed in the lines to retain large clots which, if transfused into a patient, could become gross emboli. It was of significant interest to determine if these clots of fibrin and platelets might contain DEHP and thus indicate a relationship between the plasticizer and gross clot formation. Doctor Tom Solis, Division of Surgery, Walter Reed Army Hospital, obtained ten units of blood at 21 days of storage. Each unit was filtered through a standard blood transfusion filter (Fenwal, Baxter Laboratories, Morton Grove, Ill.) and the SFP was determined both before and after filtration. No difference in SFP could be detected after such filtration, a result indicating that the filter did not remove microemboli. The 10 filter assemblies were analyzed for the amount of DEHP retained in them. It was found that 1.2 total mgs could be recovered from the ten screens, or an average of 0.12 mg of DEHP per screen. Thus, gross clots in stored human blood are documented to contain DEHP.

It became of further interest to examine units of stored platelet concentrate. These units contain approximately 70% of the platelets present in a unit of human blood, and the aggregation of platelets is known to contribute to the rise of SFP seen with storage. Similarly, the

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clots found in the blood transfusion filters also contain platelets, and DEHP. Thus, a relationship may exist between the plasticizer and increased screen filtration pressure.

Five units of two day stored platelet concentrate were obtained from the Johns Hopkins Blood bank. These units had been stored for two days in commercial PVC transfer packs (Fenwal TA-2, Baxter Laboratories, Morton Grove, Ill.) at room temperature, and with constant agitation (standard storage conditions for platelets). Each unit was entered and a ten ml aliquot of the platelet concentrate was removed. Platelets were isolated from plasma by centrifugation at room temperature, the two fractions were separated, and their volumes noted. Analysis of DEHP content was performed as described in methods.

It was found that the plasma from these units contained an average 88% of the total plasticizer recovered from each 10 ml aliquot. However, as shown in figure thirty, the concentration of DEHP in the platelet fraction was approximately two fold the concentration found in the plasma when expressed as mgs of DEHP per 100 ml of packed platelets as compared to mgs of DEHP per 100 mls of plasma. This increased concentration represents an accumulation of DEHP by the platelets over that found in the surrounding medium. The levels of plasticizer found in the platelet fraction must be due to preferential uptake or binding of the plasticizer to platelets since the centrifugation procedure used to separate the plasma from platelets would have resulted in the floatation of droplets of DEHP. Therefore, the concentration of DEHP by a fraction known to form aggregates

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Figure 30. Units of human platelet concentrate (platelet rich plasma) were obtained from the Johns Hopkins blood bank after two days of storage in commercial PVC plastic bags (Fenwal transfer pack TA-2, Baxter Laboratories, Morton Grove, Ill.). The units were stored under standard blood bank conditions for these fractions.

Aliquots of 10 ml were drawn from the bag and subjected to centrifugation. The packed cell volume of the platelets was noted, and the samples were analyzed for DEHP content as described in methods. The concentration of DEHP in terms of mgs/100 ml of each fraction was determined from the amount of plasticizer recovered from each fraction divided by the volume of that fraction times 100.

* indicates $p < 0.01$

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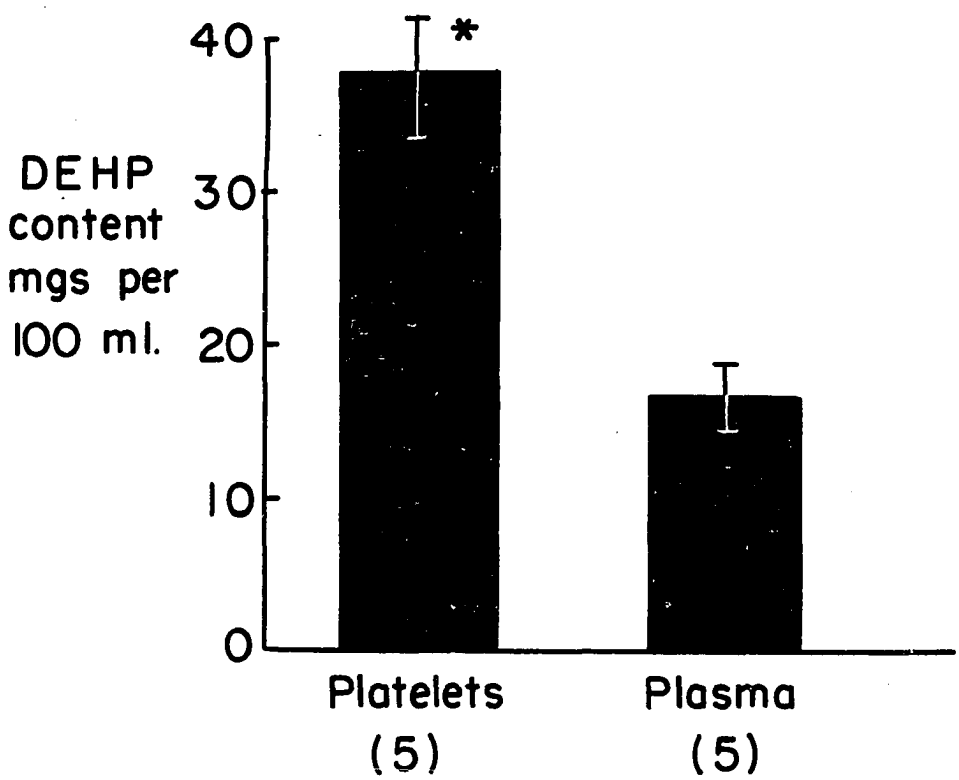
Di-2-ethylhexyl phthalate content of stored units of platelet rich plasma (human)



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and the previously shown relationship between DEHP and SFP (figure twenty-nine) suggests a possible role of this plasticizer in the formation of micro-emboli in units of stored blood.

N. Phthalate Ester Plasticizers - Effects on Isolated, Beating Heart Cells in Tissue Culture

An additional example of plasticizer toxicity was brought to the author's attention by Dr. Robert DeHaan of the Embryology Division of the Carnegie Institute of Washington. Dr. DeHaan has for some time been successfully culturing embrionic chick heart cells in serum-containing nutrient medium. Upon microscopic examination, these heart cells can be seen to be beating and, under the standard conditions used in Dr. DeHaan's laboratory, they remain beating and viable for extended periods of time. Recently, Dr. DeHaan had occasion to modify his procedure slightly; he began to feed the serum-containing culture medium into the plates containing the heart cells via a PVC plastic intravenous drip set (Plexitron R 41, Baxter Laboratories, Morton Grove, Ill.). He then observed that the beating heart cells gradually assumed an abnormal granular (blistered) appearance and that within 30 minutes, ceased beating. This was followed by a progressive decrease in the number of viable cells and finally death of all cells in the culture plate in twenty-four hours. Several experiments by Dr. DeHaan (not presented here) confirmed unequivocally that the plastic drip set was contributing the toxic factor.

This author, in collaboration with Dr. DeHaan, analyzed samples of the plastic used in the tissue culture experiments; both DEHP and DEHA

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were identified. Two types of experiments were then set up. In the first, Dr. DeHaan's standard nutrient medium was exposed to the plastic iv drip set for 30 minutes at room temperature. The medium was then removed from further contact with any plastic surface, its DEHP and DEHA content assayed, and then used undiluted, as well as in a series of dilutions with uncontaminated media, to culture beating heart cells. Each dilution was visually evaluated by Dr. DeHaan for any toxic effect. In a second parallel experiment, pure DEHP and DEHA were added directly to fresh non-plastic exposed culture media. The plasticizers were solublized in the nutrient medium by sonication. Any non-solublized plasticizer (i.e., droplets) were presumably removed when the sonicated medium was filtered through a 0.45 micron millipore filter for the purposes of sterilization. These spiked media were assayed for plasticizer content and then used undiluted to culture heart cells. Any observed toxic effect was equated to a dilution of the plastic-exposed media which produced an equivalent effect. The results of these two types of experiments are shown in table eleven.

It can be seen in table eleven that measurable quantities of both DEHP and DEHA (0.097 ug/ml and 0.044 ug/ml respectively) could be found in the tissue culture medium exposed to the plastic device for only 30 minutes. The toxicity of this undiluted medium as well as of the various dilutions is described in the table and indicates a dose related effect, the 27 fold dilution still exhibiting some toxic effect.

The three plasticizer-spiked media were also toxic to the heart cells in tissue culture. However, it should be noted that the spiked media

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Table Eleven. These experiments were conducted in collaboration with Dr. Robert DeHaan, Carnegie Institute of Washington. Samples of nutrient medium used in tissue culture experiments were exposed to PVC plastic intravenous infusion sets (Plexitron R41, Baxter Laboratories, Morton Grove, Ill.). They were analyzed for their DEHP content, and were also evaluated for their toxicity in tissue culture experiments.

Samples of plasticizer-spiked nutrient medium were prepared by sonication of the medium with either DEHA or DEHP. These were analyzed for plasticizer content and were also evaluated for tissue culture toxicity. In these experiments, control plates with non-plastic exposed medium were run concurrently with the test plates and viability and contractibility were no different than routinely observed.

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TABLE ELEVEN

PLASTICIZER EFFECTS ON ISOLATED, CHICK BEATING HEART CELLS

DILUTIONS OF PVC EXPOSED MEDIUM	PLASTICIZER CONTENT µg/ml DEHP	DEHA	TOXICITY EVALUATION
UNDILUTED	0.097	0.044	Cells cease beating in 30 minutes, become granular and blistered in 3 - 6 hrs., and are all dead in 24 hours.
1:3	0.032	0.015	Similar pattern to above but 2 - 3% survive and continue to beat at 24 hrs.
1:9	0.011	0.005	Medium not lethal to most cells. Reduces number of beating cells to 1/2, and still causes blistered appearance.
1:27	0.004	0.002	No effect on cell appearance but 1/3 reduction in number of beating cells.
MEDIUM + PLASTICIZER			
A (DEHP added)	NOT ASSAYED	0	Equivalent effect to undiluted PVC exposed medium.
B (DEHP added)	4.0	0	Equivalent to a 1:3 dilution of PVC exposed medium.
C (DEHA added)	0	1.55	Equivalent to a 1:9 dilution of PVC exposed medium.

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were toxic at concentrations some 100 - 300 times greater than that in the PVC-exposed media, e.g., solution B containing 4 μg/ml of DEHP was equi-toxic to the plastic-exposed media containing only 0. 03 μg/ml, or solution C containing 1.55 μg/ml of DEHA was equi-toxic to the plastic-exposed media containing only 0.0048 μg/ml.

Two conclusions are to be drawn from these data. The first is that the plasticizer content per se of the plastic-exposed media is most likely not sufficient to account for the observed toxicity. It is possible that in the plastic medical device used here, other substances are leached into the serum-based medium which add to any toxicity contributed by the plasticizers alone. A prime candidate for such a substance, which is known to be highly toxic to heart cells, are the heavy metals used in plastic materials as stabilizers. However, it is not the object of this study to identify the exact chemical nature of the toxic chemical in this situation. Second, the conclusion that is of greater significance relative to the overall current study is that levels of plasticizer that are highly toxic to cultured heart cells are levels that have been shown to be reached and even exceeded in plastic stored blood that is transfused into man.

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V. DISCUSSION OF RESULTS

The initial observation that forms the basis of this dissertation is that a plasticizer was extracted from PVC plastic tubing by the circulating blood in an isolated organ perfusion apparatus. The plasticizer was identified as butyl glycolylbutyl phthalate, and its conversion to glycolyl phthalate by a perfused liver has been demonstrated. A second plasticizer, di-2-ethylhexyl phthalate (DEHP) was also found to be extracted from another formulation of PVC tubing, but no metabolism of this second plasticizer to phthalic acid could be demonstrated in the isolated perfused liver.

It was further observed that this latter plasticizer was quantitatively accumulated in virtually unchanged form by the perfused liver system. So efficient was this organ accumulation that within 30 minutes after addition of an exogenous source of DEHP, the perfusate was essentially cleared of this material. Studies in the intact animal supported the observation that DEHP was not metabolized since the expected metabolite, phthalic acid was not found to be increased in the urine of DEHP treated rats. Thus, the previous reports by others of the urinary excretion of phthalic acid following DEHP were not confirmed. The apparently contradictory results were found to be due to methodologic differences The presence of increased urinary phthalic acid after DEHP treatment in the report of Shaffer et al (1945) was a result of the method of treatment of the urine prior to assay.

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Due to the observation that the isolated perfused liver accumulates DEHP from the perfusate, the possibility was investigated that this plasticizer could be accumulated by tissues other than the liver following intravenous administration in the intact animal. It was found that DEHP could be recovered in highest concentration from the lungs, liver, and spleen. However, in this experiment, after 24 hours, only 79% of the administered dose could be recovered as unaltered DEHP. While this result suggests that metabolism may occur, such metabolism, based on the results in the isolated, perfused liver and in vivo cannot occur via the formation of free phthalic acid.

Because of the observations on the extraction of plasticizers by blood and by protein-containing solutions, the possibility was investigated that human blood which is stored or circulated in PVC plastic medical devices may also be able to extract plasticizers. It was found that such extraction did occur, and two plasticizers, di-2-ethylhexyl phthalate and di-2-ethylhexyl adipate could be found in human blood that was exposed to various plastic devices in vitro. Both of these plasticizers were found in the PVC tubing of two artificial organ systems while only DEHP was found in the various blood storage bags that were tested. Having established the presence of a plasticizer in human blood under conditions in which the blood would be used in man, it became of extreme importance to determine the disposition of this contaminating material when plasticizer-contaminated blood was transfused into human beings.

It was found that DEHP was accumulated by the tissues of some

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but not all individuals who received transfusions of plasticizer-contaminated blood. The pattern of accumulation of the plasticizer from those individuals who were found to have it in their tissues was qualitatively similar to the pattern observed in the rat after intravenous administration. However, not all individuals who received amounts of the plasticizer DEHP had this material present in their tissues. This result indicated that metabolism of the plasticizer may have occurred to a greater extent in man than was observed in rat.

In order to investigate the possibility of metabolism of DEHP by man urine was collected from a single patient before and after open heart surgery, in which the patient's blood was circulated through the plastic tubing of a cardiac bypass apparatus. An increase in urinary phtalic acid was observed in this patient immediately after surgery, but the amount of the increase was not sufficient to account for the calculated amount of DEHP that he had received in blood transfusions. However, when the methods of Shaffer et al(1945) were applied, it was found that the urine now contained sufficient "phthalic acid" to account for the DEHP he may have received by transfusion, and an additional amount which had come from the tubing of the cardio-pulmonary bypass apparatus.

It was further observed in this patient that his pre-surgical urine contained measurable levels of both phthalic acid (Jaeger method) and "phthalic acid" (Shaffer method). When this latter procedure for measuring urinary metabolites of DEHP was employed in the analysis of urine from three normal persons, it was found that they each had

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significant amounts of this urinary "PA". However, adipose tissue from normal persons who had no known blood transfusions were not found to contain DEHP and it is possible that, although phthalate containing compounds, ie., DEHP may be ingested in the diet, these materials are probably metabolized and at least this one plasticizer, DEHP, does not accumulate in the adipose tissue of normal persons. However, the rate of metabolism in humans may vary from individual to individual and thus, it was possible to observe tissue levels of DEHP in some but not all persons where DEHP contaminated blood had been administered.

The plasticizing substances, DEHP, and BGBP, have been found to be without apparent toxicity in animal studies, and their lethal effects are quite minimal. However, further experiments in animals were done to determine the effects of plast i cizers on the pharmacologic and behavioral responses of the animals. It was found that the plasticizers BGBP, and DEHP were both able to prolong hexobarbital sleeping time in mice, and a dose response relationship could be shown. It was also found that rates of growth were depressed only when DEHP was administered, but the mice used in the study were able to adapt to most of the effects of the chemical. In rats, DEHP did prolong the hexobarbital sleeping time, and a possible metabolite of DEHP, 2-ethylhexanol, was also found to increase sleeping time. However, in this latter experiment, the observed effect on sleeping time would have required complete metabolism of the administered DEHP. It is unlikely that the DEHP effect on hexobarbital sleeping time that was observed in the intact animal was mediated by metabolism of the

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plasticizer to a material such as 2-ethylhexanol.

It was apparent from experiments shown that DEHP does not alter brain sensitivity to the drug hexobarbital. Further, since zoxazolamine sleeping time was not prolonged by DEHP, it may be argued that uptake of the drug from the peritoneal cavity is not affected. Further, pretreatment of the isolated perfused liver with amounts of DEHP did not alter its ability to metabolize hexobarbital. It is presently unclear how the compound DEHP is able to prolong hexobarbital sleeping time in both rats and mice.

During the experiments reported here, it was found that after intraperitoneal injection of DEHP into rats, large white masses could be found adherent to the omentum and various other abdominal organs. When these masses were examined by a vetrinary pathologist, he concluded that they were indicative of an exudative peritonitis associated with the ip administration of an irritating substance. Murphy (1969) has shown that the irritant, acrolein, causes a stress responsein rats after ip injection which results in an elevated plasma corticosterone level. Barnes and Wooles (1970) have shown in mice that the intravenous administration of corticosterone immediately prior to hexobarbital results in an enhanced sleeping time. Presumably, the steroid is acting as a competitive inhibitor of hexobarbital metabolism in vivo. Thus, it may be possible that DEHP is able to cause a prolonged sleeping time in rats and mice due to the stress response that it may cause. Because the possibility of such a response is lacking in the isolated perfused liver, DEHP should have no

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effect on the rate of drug metabolism and this indeed was the observed result.

It is not clear why a difference exists between the in vivo response to hexobarbital and zoxazolamine after DEHP pretreatment. Both compounds are metabolized by the liver, but it is possible that their metabolism has different sensitivity to the possible stress mediated effects of DEHP.

In further studies on the effects of DEHP after its intraperitoneal administration, it was found that two forms of behavior were markedly altered. The operant conditioned responses of rats to hypothalamic shock were severely depressed as was their spontaneous running activity. Both of these decrements in behavior were associated with marked losses in body weight, similar to the effect seen in mice after a single ip injection of 250 mgs/kg of DEHP. It was further shown that the loss in body weight was due to a decreased food and water consumption in the 24 hours which followed the plasticizer dose. It is possible that all four of these observations may be explained on the basis of intraperitoneal irritation, and a decreased activity of the animal in response to pain. It would be unlikely that an animal with very severe abdominal pain would eat or drink during this time, nor would he be expected to be as active or as responsive as a normal animal might be.

It is interesting to note, however, that a weight loss was observed in rats during the 24 hours after an intravenous dose of DEHP. The amount of weight lost by these animals was only 2% of their body weight, but the

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loss was significantly different from control. While the cause of the observed weight loss is not yet known, it may be related to the irritant properties of DEHP. It is interesting to speculate that the intravenous administration of an irritant substance may have a similar but less pronounced effect than is seen after intraperitoneal administration of the material.

The solutions of DEHP used in these experiments were aqueous emulsions, and it has been shown by others that emulsified lipids and particles are able to alter the carbon clearance from the blood by the reticulo-endothelial system. When DEHP was tested in this regard, it was found that a single dose of the plasticizer could decrease the rate of carbon clearance when this function was measured 24 hours after the iv dose of DEHP. Repeated smaller doses were also found to alter the carbon clearance but these doses caused the rate to be enhanced slightly (second experiment only). It is not known what effect these treatments might have on the rats ability to withstand the effects of infections but it has been shown by other workers (Horn, et al 1969) that the RE system removes bacteria by a process of phagocytosis. A decreased rate of particle clearance might then result in an increased bacterial activity and an enhanced likelihood of infection.

The results that have been discussed to this point have been directed toward the effects on the intact animal of plasticizers in general and DEHP in particular. Of equal significance is the possible effects that extracted plasticizers might have on isolated cells such as those in

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stored blood which may be exposed to concentrations of the plasticizer, DEHP. One example of blood cell damage was first reported by Swank (1961), and this effect is the tendency of platelets to aggregate on storage. His original observations were made on blood which had been stored in glass and thus, plasticizers could not be involved. However, the mechanical trauma which results from the blood having been subjected to a vacuum, and the denaturating effect of a residual air-blood interface may have caused the observed platelet aggregation. Further, glass surfaces are thought by some to be injurious to blood platelets (Ozge, et al, 1964).

It was found that platelet aggregation occurred in PVC plastic blood bags, systems that had none of mechanical or surface problems of glass. It was also observed that alteration of the PVC surface did cause a reduced amount of platelet aggregation as measured by the screen filtration pressure. This alteration involved addition of heparin to the walls of the bag, and while it reduced the rate of increase of screen filtration pressure, this treatment did not affect the rate of plasticizer elution into blood. Thus, it is not yet clear what effect the contaminant DEHP has on platelet aggregation and screen filtration pressure. If the effect of the plasticizer is a direct one, the addition of a heparinized wall surface appears to reduce the degree of platelet aggregation. However, as was mentioned previously, the rate of increase of screen filtration in this heparinized container significantly correlated with DEHP content.

Some units of blood were stored in an experimental plastic,

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Hemosil, 103. This material permitted the gentle collection of blood, and more important, it did not contain plasticizers or any additives. During storage, it was found that blood from these bags had no change in screen filtration pressure with time, a result which indicates that damage to the platelets may have been prevented altogether. It is, at present, not known whether this protection of platelets in Hemosil 103 is due to differences in wall surface of the plastic bag or the absence of a plasticizer.

In an attempt to further elucidate the possible reasons why PVC stored blood develops aggregates, units of human platelet rich plasma were analyzed for their DEHP content. It was found that the platelets accumulate the plasticizer to a higher concentration than is found in the surrounding plasma. These units of platelet rich plasma are no longer useful for transfusion after two days of storage at 22°C. It is not presently known why their viability is so limited, but the observation that a fraction of blood is known to form platelet aggregates and is able to accumulate DEHP to a high concentration may be indicative of some detrimental effects of this plasticizer on stored human platelets.

Further experiments were performed on isolated beating heart cells in tissue culture. This work, done in collaboration with Dr. Robert DeHaan, indicated that the plasticizers DEHP and DEHA were both toxic to beating heart cells at concentrations found in one day old units of stored human blood, or blood that had circulated in PVC plastic tubing. The possibility exists that isolated beating heart cells in tissue culture may be much more sensitive to the toxic effects of plasticizers than other cells

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exposed to plastics under different conditions, e.g., human platelets. However, the decreased viability of isolated human platelets and the accumulation of DEHP in these cells leads the author to further question the possible role of extracted plasticizers in reduction of cell viability with storage.

Also, it is not yet known if blood levels of certain plasticizers, e.g., DEHP, have any effects on the cells of human tissue in vivo after extensive transfusion. Even minimal effects on cardiac and other tissues may have the gravest consequences in those individuals who receive blood transfusions during periods when their ability to withstand toxic stress is reduced by prior disease or trauma.

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VI. IMPLICATION OF RESULTS

It must be concluded that the evidence presented in this dissertation does not indict plasticizers as the cause of any ill effects seen after exposure of humans to plastic devices. However, this does not imply that no effects have occurred, but rather, the plasticizer has not been implicated in the sequelae of such exposure.

McNamara et al (1970) in his studies on the pulmonary function of combat casualties after massive transfusions has shown that screen filtration pressure in circulating blood is increased. Further, these patients have respiratory problems which are highly correlated to the number and age of the units of blood that he has received. Further, cardio-pulmonary bypass has been shown to cause changes in pulmonary function. The changes are: altered ventilation-perfusion relationships (Hedley-Whyte, et al, 1965), decreased compliance of the lung with increased respiratory work (Garzon, et al, 1966), and changes in oxygen diffusing capacity (Anderson and Ghia, 1970).

Recently, CPB has been found to cause certain neurologic changes and these are described by Rimon et al (1970). The observed symptoms are: confusional states, depressions, anxiety, and psychomotor disturbances. Similarly, hemodialysis has some neurologic changes associated with it and these have been reported by Tyler (1965). He reports that mental confusion and schizoid symptoms are observed after long runs on dialysis. Further, during dialysis, the patient may become drowsy and find concentration to be difficult. As dialysis

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is prolonged, the patient may become irritable. Headache, nausea and, rarely, vomiting are also seen. Convulsions and coma may occur but these symptoms are aborted if dialysis is discontinued. Further, neuropathies, both sensory and peripheral, may develop with chronic hemodialysis.

In studies of hemodialysis, cardio-pulmonary bypass, and massive blood transfusion, the question of extraction, metabolism or accumulation of plasticizers has not been considered. No coordinated effort has ever been made to determine if there was any relationship between the symptoms observed and the possible role of the plastic materials used in the procedures. The results of this dissertation are sufficient evidence to warrant this consideration.

Further, the effects of plastics and extractable materials must also be considered from the standpoint of isolated cells in a closed system. It was shown by Ozge et al (1964) that PVC materials were detrimental to certain platelet functions, i.e., clot retraction and serotonin uptake. He was able to show that these functions were irreversibly affected by storage in PVC plastic, but the reasons for the effect were not clear. More recently, Murphy and Gardner (1969) have shown that platelet viability is better preserved by storage at 22° rather than at 4° as used by Ozge et al (1964). However, in their most recent work Murphy et al (1970) have shown still further improvement in the storage of platelets when a change in the PVC plastic material of the storage bags is made. This new material is claimed by the manufacturer to contain the same

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amount of DEHP as the older material but the incorporation of an undisclosed additive results in a decreased leachability of the plasticizer DEHP (Gesler, 1970., personal communication). It is not yet apparent whether this decreased leachability is solely responsible for the improvement in platelet storage life, but it does indicate that the effect of the extracted plasticizer on stored platelets may be an important one.

Further investigation of the role of extracted plasticizing substances from PVC plastic medical devices is indicated. Careful consideration should be given to the effects that these compounds have on human beings who are exposed to them. In addition, the effects of extracted plasticizers on the storage life and viability of isolated cells must be considered. Factors such as economy and convenience do not represent sufficient cause to warrant continued use of materials which contribute contaminants which may be even slightly hazardous.

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