References and Notes

1. A. Dorfman and Pei-Lee Ho, *Proc. Nat. Acad. Sci. U.S.* **66**, 495 (1970).
2. H. V. Castejón, *Acta Histochem.* **35**, 161 (1970).
3. J. D. Robinson and J. P. Green, *Yale J. Biol. Med.* **35**, 248 (1962).
4. B. S. McEwen and B. Grafstein, *J. Cell Biol.* **38**, 494 (1968).
5. J. S. Elam and B. W. Agranoff, *J. Neurochem.*, in press.
6. M. Singh, E. V. Chandrasekaran, R. Cherian, B. K. Bachhawat, *ibid.* **16**, 1157 (1969); K. Saigo and F. Egami, *ibid.* **17**, 633 (1970).
7. D. Lagunoff and G. Warren, *Arch. Biochem.* **99**, 396 (1962).
8. W. L. Cunningham and J. M. Goldberg, *Biochem. J.* **110**, 35P (1968).
9. S. Ochs and J. Johnson, *J. Neurochem.* **16**, 845 (1969).

10. We thank Mrs. I. Mason and Mrs. M. Andrews for technical assistance and Dr. C. W. Castor for a gift of standard sulfated mucopolysaccharides. Supported by grants from NSF, NIH, and NIMH.
* Interdisciplinary fellow of NIMH training grant MH 07417.

16 July 1970

Plasticizers from Plastic Devices: Extraction, Metabolism, and Accumulation by Biological Systems

Abstract. *Phthalate ester plasticizers were found to be extracted by blood from plastic tubing and from plastic bags used for blood storage. One such plasticizer was metabolized by the isolated perfused rat liver while another was found to be accumulated in the liver unchanged. In addition, this latter plasticizer was identified in samples of human tissue taken from patients who had received transfusions of blood stored in plastic bags. The biological implications of these observations are considered.*

In experiments in which the technique of the isolated, perfused rat liver was used, three acidic ultraviolet-absorbing materials were observed to accumulate in the plasma of the perfusion medium. In Fig. 1 are shown the elution patterns from the chromatographic fractionation of acid-soluble extracts of perfusion plasma which had either perfused a liver or had circulated in the apparatus in the absence of a liver. The first peak (I) is common to both experimental conditions and on the basis of its ultraviolet spectrum and elution volume was identified as uric acid. Peak II, a compound formed only when a liver is present, has not yet been identified. Peak III, which also appears only when a functioning rat liver is present in the perfusion system, was selected for further identification.

The unknown material in peak III was extracted from acidified aqueous solutions with diethyl ether. Furthermore, treatment of the ether-extractable material with diazomethane, a methylating agent, led to a loss of the compound's water solubility. These results suggested the presence of an organic acid. The methylated derivative was subjected to nuclear magnetic resonance spectroscopy and mass spectrometry. Analysis of the spectrophotometric data led to the tentative conclusion that the unknown molecule was an ester of glycolic acid and phthalic acid, glycolyl phthalate (GP), as shown in Fig. 2.

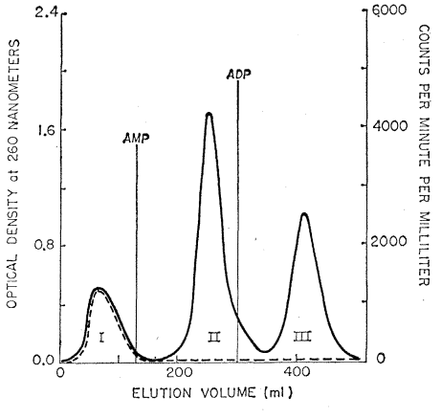

Fig. 1. Circulation of perfusion fluid in the presence (———) and the absence (- - - - -) of a liver. The perfusion fluid, a mixture of 70 ml of whole rat blood containing 70 units of heparin per milliliter and 35 ml of Krebs-Ringer-bicarbonate buffer containing 4 percent BSA and 80 mg of glucose per 100 ml, was circulated in the liver perfusion apparatus of Miller *et al.* (*17*). This system was used to perfuse an isolated rat liver for 4 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 2*N*, *p*H 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.

As there is little information on the biological origin of phthalates and since it is known that esters of phthalic acid are used as plasticizers in the formulation of various plastics (*1*), the possibility was investigated that the GP arose as a metabolite of a plasticizer extracted from the polyvinyl chloride tubing used in the perfusion apparatus. The manufacturer of the tubing (*2*) identified the plasticizer as butyl glycolylbutyl phthalate (BGBP). Thin-layer chromatography of organic extracts of perfusion plasma, which had circulated in the apparatus in the absence of a liver, revealed appreciable quantities of BGBP.

In Fig. 3 is shown the time course for the accumulation of GP in the perfusate under three experimental conditions: (i) experiment A, in which a large quantity of BGBP was added exogenously to the perfusate at time zero; (ii) experiment B, in which polyvinyl chloride tubing was the only source of BGBP; and (iii) experiment C, in which ether-washed gum rubber tubing was used. It can be seen from experiment C that in the absence of exogenously added or endogenously available BGBP, no GP can be detected, while experiments B and A demonstrate that the polyvinyl chloride tubing or exogenously added BGBP leads to accumulation of GP. Thus it is concluded that BGBP is extracted from plastic tubing by blood, is metabolized by the liver, and its product GP is secreted into the perfusion medium.

A second phthalate ester plasticizer commonly encountered in plastics used in biological and medical practice is di(2-ethylhexyl)phthalate (DEHP) (*3*). Tubing formulated with this plasticizer (*4*) was also tested for extraction of the phthalate ester by the perfusion medium. Significant levels of DEHP (0.01 to 0.05 mg per milliliter of plasma) could be detected in perfusion fluid that had circulated through this type of tubing in the perfusion apparatus in the absence of a liver. As was the case with BGBP, no DEHP was seen in the per-

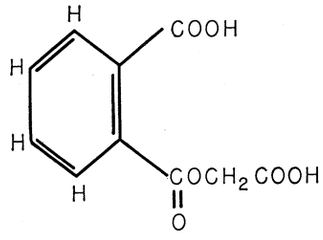

Fig. 2. Glycolyl phthalate (GP).

fusate when a liver was present in the system. However, in contrast to the situation with BGBP, in which the plasticizer was recovered as the water-soluble metabolite, in the case of DEHP no trace of a de-esterified metabolite, that is, phthalic acid, could be found. Even after the exogenous addition of DEHP (0.3 mg per milliliter of plasma) to the blood perfusing a liver, phthalic acid could not be detected. This lack of an observable metabolite occurred in the face of a complete clearance of the added DEHP from the perfusion medium within 60 minutes after addition. Analysis of the liver showed that, at this time, approximately 90 percent of the total recoverable plasticizer was sequestered in the liver. Thus, it is apparent that DEHP, unlike BGBP, is not de-esterified by the isolated rat liver, but rather is accumulated by that organ, primarily in unmetabolized form.

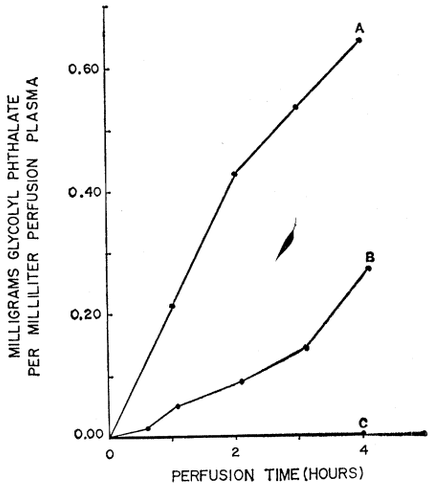

Fig. 3. Perfusion experiments were done as outlined above. The content of glycolyl phthalate was determined as follows. In experiment A, after extraction of the plasma with chloroform at neutral *p*H to remove lipid-soluble material, a portion of the remaining aqueous phase was fractionated by thin-layer 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.

The extractability of plasticizers from plastic items was explored further. Thus, simple salt solutions (such as physiological saline) were found to be unable to extract DEHP from polyvinyl chloride tubing, even after 6 hours of recirculation in the tubing. On the other hand, a 4 percent bovine serum albumin (BSA) solution was found to extract approximately 40 percent of the amount of plasticizer extractable by whole blood (*5*). In addition, samples of human blood were obtained from two blood bank services. The blood, of various types, had been stored in DEHP plasticized polyvinyl chloride blood bags at 4°C for periods up to 21 days. Analysis of these samples showed that the DEHP content increases with time of storage and reaches a level of 5 to 7 mg of plasticizer per 100 ml of blood at the end of the 21-day expiration period (*6*). Fractionation of the blood indicated that the plasticizer was located almost entirely in the plasma fraction and was specifically associated with the lipoprotein fraction of plasma.

Preliminary experiments with tissue obtained from two patients who had received blood transfusions while at the Johns Hopkins Hospital indicated that spleen, liver, lung, and abdominal fat all contained significant quantities of DEHP, ranging from 0.025 mg/g (dry weight) in spleen to 0.270 mg/g (dry weight) in abdominal fat (*6*).

While the precise biological implications of these findings have yet to be determined, the ubiquitous use of plastics and plasticizers in the environment today should give rise to a heightened awareness about the biochemical and toxicological role of these types of compounds in man and animal. In addition to published reports of the identification of phthalate ester plasticizers in certain foodstuffs, such as milk (*7*) and fat from the deep fryer of a drive-in restaurant (*8*), there has been evidence concerning the presence of these types of chemicals in animal tissues, such as beef pineal gland (*9*) and heart (*10*). Guess (*11*) has reported the isolation of DEHP from the anticoagulant citric acid–dextrose solutions stored in disposable polyvinyl chloride blood bag assemblies, and a recent study (*12*) has shown the presence of another phthalate ester in blood stored in bags made of the same material. In this report we document the presence of a plasticizer in human tissue.

In general, phthalate ester plasticizers have been reported to have a low order of toxicity in experimental animals (*13*) and, thus, are approved by the Food and Drug Administration for use in packaging materials for food intended for human consumption (*14*). However, the sanction of the use of this type of compound is based on the absence of overt toxic symptoms following oral administration. In recent years Guess and co-workers (*15*) have published a series of papers dealing with the "subtle toxicities" of plasticizers, stabilizers, and so forth, used in the manufacture of polyvinyl plastics. Examples of this type of toxicity are tissue culture cell death or enhanced growth, changes in antibody reactivity, and irritation as evidenced by dye extravasation. It is in this area of subtle toxicity, possibly associated with accumulation and metabolism of plasticizers, that further investigations are required. The importance of such investigations are particularly emphasized when one considers the increasing use of plastics in medical, pharmaceutical, and cosmetic devices over which there currently exist no federal control or regulations (*16*).

RUDOLPH J. JAEGER
ROBERT J. RUBIN
Department of Environmental Medicine, School of Hygiene and Public Health, Johns Hopkins University, Baltimore, Maryland 21205

References and Notes

1. C. R. Noller, *Chemistry of Organic Compounds* (Saunders, Philadelphia, ed. 3, 1965), p. 603.
2. Tygon tubing formulation S-22-1; Norton Co., Akron, Ohio 44309.
3. DEHP is frequently, but erroneously, referred to as DOP (di-octyl phthalate).
4. Tygon tubing formulation S-50-HL; Norton Co., Akron, Ohio 44309.
5. R. J. Jaeger and R. J. Rubin, *Fed. Proc.* **29**, 411 (1970).
6. ———, *Lancet* **1970-II**, 151 (1970).
7. J. Cerbulis and J. S. Ard, *J. Ass. Offic. Anal. Chem.* **50**, 646 (1967).
8. E. G. Perkins, *J. Amer. Oil Chem. Soc.* **44**, 197 (1967).
9. R. G. Taborsky, *J. Agr. Food Chem.* **15**, 1073 (1967).
10. D. J. Nazir, M. Beroza, P. P. Nair, *Fed. Proc.* **26**, 412 (1967).
11. W. L. Guess, J. Jacob, J. Autian, *Drug Intelligence* **1**, 121 (1967).
12. Y. L. Marcel and S. P. Noel, *Lancet* **1970-I**, 35 (1970).
13. H. C. Hodge, *Proc. Soc. Exp. Biol. Med.* **53**, 20 (1943); C. B. Shaffer, C. P. Carpenter, H. F. Smyth, Jr., *J. Ind. Hyg. Toxicol.* **27**, 130 (1945); C. P. Carpenter, C. S. Weil, H. F. Smyth, *Arch. Ind. Hyg. Occup. Med.* **8**, 219 (1953).
14. Food additives amendment to the Food, Drug, and Cosmetic Act, Subpart E-121.2001, *Federal Register* (15 Oct. 1968), 33 F.R. 15281.

15. W. L. Guess and S. Haberman, *J. Biomed. Mater. Res.* **2**, 313 (1968); J. Nematollahi, W. L. Guess, J. Autian, *J. Pharm. Sci.* **56**, 1446 (1967); W. L. Guess, S. Haberman, D. F. Rowan, R. K. Bower, J. Autian, *Amer. J. Hosp. Pharm.* **24**, 495 (1967); D. Calley, J. Autian, W. L. Guess, *J. Pharm. Sci.* **55**, 158 (1966); D. B. Myers, J. Autian, W. L. Guess, *ibid.* **53**, 774 (1964).
16. J. Autian, H. Berg, W. L. Guess, *Soc. Plastic Engineers, 22nd Annual Technical Conference, Tech. Papers XII*, XIX-3 (1966).
17. L. L. Miller, C. G. Bly, M. L. Watson, W. F. Bales, *J. Exp. Med.* **94**, 431 (1951).
18. We gratefully acknowledge the help and advice of C. C. Fenselau, C. H. Robinson, and S. Y. Wang, as well as the excellent technical assistance of Donald Brown and Laurie Butler. This work was supported by PHS grants ES 34-07 and AM 9392-06 and by career development award ES 4488-01 to R.J.R., and is submitted in partial fulfillment for the Ph.D. degree (R.J.J.).

3 August 1970

Radioresistance of Cooperative Function of Carrier-Specific Lymphocytes in Antihapten Antibody Responses

Abstract. *Transfer of lymphoid cells from strain-2 guinea pigs immunized to bovine gamma globulin into syngeneic recipients immunized with dinitrophenyl ovalbumin markedly enhances the secondary antidinitrophenyl response of the recipient to challenge with dinitrophenyl–bovine gamma globulin. This function of the carrier bovine gamma globulin–specific cells is resistant to irradiation with up to 5000 rads, although the capacity of the irradiated cell population to transfer immunologic memory for bovine gamma globulins or to be stimulated by antigen to synthesize DNA in vitro is abolished by this treatment.*

The production of hapten-specific antibodies in response to immunization with haptenic carrier conjugates can be markedly enhanced by preimmunization of animals with unmodified carrier. This can be demonstrated strikingly in secondary antihapten antibody responses to hapten conjugates of a protein not used in primary immunization (*1–3*). Furthermore, preimmunization with carrier enhances the primary antihapten antibody response to a hapten-carrier complex as well (*3*). These findings suggest the existence of independent recognition units for hapten and for carrier determinants, the cooperation of which is of considerable importance in the development of hapten-specific immune responses.

It has been amply demonstrated that the functional carrier recognition unit is not conventional humoral antibody (*2, 3*). In the adoptive secondary antibody response in mice, Mitchison and his collaborators have shown that cooperative interactions are mediated by hapten-specific and carrier-specific lymphoid cells (*2, 4*). More recently we have demonstrated that transfer of live lymphoid cells from strain-2 guinea pigs immunized with bovine gamma globulin (BGG) to syngeneic recipients previously primed with 2,4-dinitrophenyl-ovalbumin (DNP-OVA) enhances anti-DNP antibody responses of these recipients to subsequent challenge with DNP-BGG (*5*). Thus, in both species the carrier recognition unit appears to be associated with specific lymphoid cells. In this report we present data showing that the carrier function of these lymphoid cells is radioresistant.

The experiments were carried out in strain-2 guinea pigs. Donor guinea pigs were immunized in the four footpads with either 50 μg of BGG emulsified in complete Freund's adjuvant (CFA) or saline emulsified in CFA. Three weeks later the animals were killed and axillary, occipital, inguinal, and popliteal lymph nodes and spleen were removed. Single cell suspensions, in minimum essential medium (Eagle), were prepared and washed. In each experiment, cell suspensions from BGG cell donors and CFA cell donors were pooled separately. Portions of the pooled cells were either irradiated (*6*) or not irradiated. Portions not irradiated were nevertheless subjected to identical conditions of handling employed for cell portions receiving irradiation, such as length of time exposed to ambient temperature. Subsequent to all manipulations, cell viability was 70 percent for both irradiated and nonirradiated cell populations, as shown by trypan blue exclusion.

Varying numbers of these cells were injected intravenously into syngeneic recipients which had been immunized 3 weeks earlier with three daily doses of 1.0 mg of DNP_7-OVA (*7*) administered intraperitoneally in saline. Recipients were given a booster dose 6 days after cell transfer with 1.0 mg of DNP_{28}-BGG in saline (200 μg intradermally followed by 800 μg intraperitoneally 4 hours later) (*8*). Animals were bled just prior to secondary immunization (day 0) and 4, 7, and 11 days later. Serum anti-DNP antibody levels were determined in a modified Farr assay by using tritiated DNP-epsilon-aminocaproic acid (*3, 9*). Quantitative determination of precipitating anti-BGG antibody was performed with I^{125}-labeled BGG as previously described (*3, 10*).

Table 1 presents the results from two experiments in which recipients received 1.2 to 1.3 $\times$ 10^9 donor cells. In the first experiment, there was a marked enhancement of the anti-DNP responses in recipients which received either nonirradiated BGG cells or BGG cells irradiated with 1500 rads as compared with responses of recipients

Table 1. Effect of in vitro irradiation of carrier-specific cells on their capacity to enhance hapten-specific anamnestic responses. N.D., not determined.

Protocol*		Animals (No.)	Anti-DNP antibody (μg/ml)†				Anti-BGG antibody (P_{50})†		
Specificity of cells transferred	Irradiation (rads)		Day 0	Day 7	Day 11	"Boost"	Day 0	Day 7	"Boost"
			Experiment 1						
BGG	None	3	0.52	63.8	108.3	107.8	47.6	82.5	34.9
BGG	1500	4	1.0	110.7	264.6	263.6	<5	<5	0
CFA	None	5	0.82	0.66	16.8	16.0	<5	<5	0
			Experiment 2						
BGG	None	4	1.1	25.7	254.3	253.2	48.4	80.6	32.2
BGG	1500	4	1.2	12.4	148.4	147.2	<5	<5	0
BGG	5000	4	0.94	2.3	47.5	46.6	<5	<5	0
CFA	None	4	.58	0.34	8.3	7.7	N.D.	N.D.	

* 1.2 to 1.3 $\times$ 10^9 donor lymph node and spleen cells were transferred to each recipient which had been previously immunized with DNP_7-OVA. Recipients were given booster doses with DNP_{28}BGG 6 days after transfer. † The data are expressed as geometric means. "Boost" represents the increase in mean antibody levels from day 0 to day 11 for anti-DNP and from day 0 to day 7 for anti-BGG. A comparision of the geometric mean "boosts" in serum anti-DNP antibody concentrations, from day 0 to day 11, gave the following results (determined by Student's *t*-test): In experiment 1 comparison of the groups receiving nonirradiated and irradiated BGG cells with the group receiving CFA cells yielded *P* values of $.05 > P > .02$ and $P < .01$, respectively. In experiment 2, comparison of groups receiving nonirradiated, 1500 rads, and 5000 rads irradiated BGG cells with the group receiving CFA cells yielded *P* values of $P < .01$, $P > .01$, and $.02 > P > .01$, respectively.