ACUTE AND SUBACUTE TOXICITY OF DI(2-ETHYLHEXYL) PHTHALATE WITH NOTE UPON ITS METABOLISM*

C. Boyd Shaffer, Charles P. Carpenter and Henry F. Smyth, Jr.[1]

Mellon Institute, Pittsburgh, Pa.

Di(2-ethylhexyl) phthalate, available under the trade-mark name of "Flexol" Plasticizer DOP, is being widely used in the production of flexible films, tubing, and the like from various synthetic resins. The commercial product is a light-colored fluid of medium viscosity with a specific gravity of 0.985 and a vapor pressure at room temperature of less than 0.01 mm. of mercury. The ester is practically insoluble in water but miscible with most organic solvents.

The communication of Hodge (1) presents the only published observations upon the toxicity of this compound. He failed to kill white rats with oral doses of 34.5 gm./kg., but did kill a small proportion of the mice given intraperitoneal injections of up to 128 gm./kg. Fatal doses to the latter species produced enlarged livers in which cloudy swelling was seen. In view of the paucity of information available by which one could judge the health hazards involved in the use of di(2-ethylhexyl) phthalate the study reported upon here was undertaken.

Single Doses

Single doses of di(2-ethylhexyl) phthalate were administered by stomach tube to male Wistar strain albino rats, weighing 90 to 120 grams and to male albino rabbits weighing 2.5 to 3.5 kilograms. The animals were observed for 14 days following the dose and those which did not die gained weight at a normal rate. The LD_{50} calculated by the methods of Bliss (2) was 30.6 (20.8 to 45.2) gm./kg. for rats and 33.9 (25.2 to 45.7) gm./kg. for rabbits. The slopes of the log-dosage vs. probit-mortality lines were 1.92 and 3.42 respectively and these low values account for the large standard deviations indicated by the fiducial limits shown in parentheses.

The plasticizer was actually administered as a dispersion in a 1 per cent solution of "Tergitol" 7 penetrant,[3] 1 ml. of which contained 0.50 gm. of the phthalate. It is our experience that administration in this form may increase intestinal absorption of water-insoluble fluids and accordingly reduce the apparent LD_{50} upon comparison with doses given undiluted or dissolved in a digestible oil. However, the largest dosage given to rats, 79.5 gm./kg., was administered undiluted and it killed 8 of the 10 animals receiving it. This mortality is in agreement with the curve representing all other results. In view of that fact we believe the surface active agent used to disperse the chemical had little if any effect upon the accuracy of the toxicity data recorded above.

Death from oral doses was delayed. Inasmuch as half of the victims survived the dose by 6 days or more, some injury other than narcosis was obviously involved in the fatalities. Histological examination of organs of the rats revealed marked generalized cloudy swelling of the liver and a moderate degree of cloudy swelling of the kidney accompanied by granular secretion in the tubules. These changes indicate that liver and kidney injury contributed to the fatal outcome.

Single intraperitoneal injections of undiluted di(2-ethylhexyl) phthalate were made in rats of the same specifications. By this route the LD_{50} was 30.7 (23.2 to 40.6) gm./kg. The peritoneal cavity of victims contained the same milky emulsion seen by Hodge (1). This fluid gave a positive test for phthalates and is believed to contain unabsorbed ester. Because the LD_{50} for rats following intraperitoneal doses was identical with that from oral doses, it appears that absorption of the ester from the digestive tract was no more complete than from the peritoneal cavity. Incomplete absorption was also inferred from the study of metabolic products in rabbits and man.

Using a minor modification of the Food and Drug Administration's cuff test (3), single doses were held upon the intact rabbit skin for 24 hours and the animals were observed for the following 14 days. The greatest dosage we are able to administer reliably by this technique is 20 ml./kg. That amount killed 2 of 6 rabbits. Hence the LD_{50} by skin absorption in rabbits is in the neighborhood of 25 ml./kg., not significantly different

* Received for publication February 19, 1945.

[1] With the assistance of other members of the Multiple Industrial Fellowship on Chemical Hygiene.

[3] Trade mark description of a surface active agent, the sodium sulfate derivative of 3,9-diethyl tridecanol-6.

from the oral LD_{50} for the same species. Therefore we conclude that skin penetration in rabbits is approximately as complete as absorption from the digestive tract. No injury to the skin resulted from these dosages.

Inhalation of Mist

Plasticizers are often incorporated into synthetic resins by milling upon hot rolls. This practice may result in a mist in the nearby atmosphere formed by condensation of plasticizer vaporized from the hot mixture, and if the plasticizer is easily oxidized its oxidation products will also be present. To effect a rough practical evaluation of

TABLE 1

Comparison of Plasticizer Health Hazards on the Basis of Inhalation by Groups of 6 Rats of Mist Formed by Cooling Air Saturated with Vapors at 170° C.

Plasticizer	Maximum inhalation period resulting in no fatality	Minimum inhalation period resulting in all dying
Diethyl phthalate	7.5 min.	15 min.
Triethylene glycol di(2-ethylbutyrate) ("Flexol" Plasticizer 3-GH)	15 min.	30 min.
Triethylene glycol di(2-ethylhexoate) ("Flexol" Plasticizer 3-GO)	30 min.	1 hr.
[illegible]	30 min.	1 hr.
Diethyl sebacate	30 min.	1 hr.
Liquid petrolatum	1 hr.	2 hrs.
Tricresyl phosphate (free from ortho)	[illegible]	[illegible]
Di(2-ethylhexyl) phthalate	[illegible]	[illegible] hrs.

health hazards of this operation, we exposed groups of 6 rats each to mists formed by bubbling a stream of air through the plasticizer maintained at 170° C. in a fritted glass gas washing bottle. The air was cooled to room temperature before entering the exposure chamber. This method results in inhalation of a mist whose concentration is of the same order of magnitude as that of air saturated with vapors at 170° C. and there is ample opportunity for oxidation products to form in the process. The exposure periods of different groups of rats were arranged in a geometrical series with a constant ratio of two. It is obvious that such an exposure is greatly in excess of that of human workmen operating mixing rolls and that it throws no light upon possible cumulative effects of repeated action. Table 1 shows the results of mist exposures using some plasticizers and other familiar fluids known to be employed with safety, for comparison with di(2-ethylhexyl) phthalate.

We recognize that the chemicals listed in Table 1 injure the body by diverse mechanisms and that they differ materially in vapor pressure. The data offer no more than a comparison of the fatal effects upon rats in an extremely severe exposure which bears qualitative similarities to a human exposure frequent in the blending of plastic mixtures. They show only that the health hazards of di(2-ethylhexyl) phthalate in this mixing process are no greater than those of some other materials already used without injury to workmen, provided its cumulative action is no greater.

Subacute Toxicity

Over a period of 90 days groups of 5 male albino rats weighing 120 to 150 gm. received daily oral doses of di(2-ethylhexyl) phthalate. The chemical was administered at levels of 3.0, 1.5, 0.75, 0.375, and 0.0 per cent mixed in a diet of ground wheat, dried milk, and salt. A supplement of liver was given twice weekly.

The amount of diet eaten was determined three times a week, and the rats were weighed weekly so that a calculation of dosage in terms of gm./kg. was possible. These dosages were 1.9, 0.9, 0.4, 0.2, and 0.0 respectively. Blood counts were performed four times, and micropathological examination was made of heart muscle, liver, kidney, spleen, and testis from all animals.

No rat died as a result of the doses. Growth of rats receiving the three greatest dosages was somewhat retarded when compared with that of the control group. The blood cell counts, hemoglobin concentrations and differential white cell counts remained normal. The only micropathology detected was tubular atrophy and degeneration in the testes, resembling senile changes, which occurred in all rats eating the two higher concentrations in the diet and in none of the other animals.

In summary, a dosage of 0.4 gm./kg./day for 90 days caused no injury to rats beyond somewhat retarded growth while a dosage of 0.2 gm./kg./day caused no injury whatever. It is impossible to translate these data to human dosages, but it is apparent that di(2-ethylhexyl) phthalate does not possess great cumulative action.

Irritation

The irritation of di(2-ethylhexyl) phthalate is low, for 0.5 ml. of the undiluted chemical in the eye of the rabbit produced no necrosis of the cornea detectable by fluorescein staining and only transient congestion of the lids. Upon the rabbit skin no congestion or other reaction resulted from a single application. The plasticizer was applied undiluted to 23 human subjects in the form of patch tests upon the back, left in contact 7 days and reapplied on the same spots 10 days later. No erythema or other reaction resulted at any time during this test. Accordingly we can say that the sensitizing power of this ester is not great.

Metabolism

No rise in the urinary excretion of glycuronic acid, ethereal sulfate, nor conjugated amino acids was observed in rabbits and rats after feeding of di(2-ethylhexyl) phthalate. These results are in accord with those of earlier investigators (4, 5). The absence of these conjugation products of phthalic acid coincides with Quick's theory (6) that the conjugation process is primarily a means of changing a weak acid which the body cannot excrete to a strong acid which it can eliminate, and that strong acids are generally excreted uncombined. Phthalic acid is more highly ionized than mandelic acid, which is known to be excreted readily unconjugated.

A relatively large increase in the excretion of fatty acids was found in the urine of rabbits receiving a single oral dose of 2 gm./kg. of the ester, following the procedure described by Friedemann (7). From a consideration of the rate of steam distillation of the fatty acid content, it appears that at least 40 per cent is made up of shorter chain acids than 2-ethylhexoic. This elevated level of fatty acid excretion was maintained for several days following the dose.

Quantitative data on the phthalate content of the urine was obtained on three species, rabbit, dog, and man, following single oral doses of di(2-ethylhexyl) phthalate. In the case of the rabbit, at least, it is presumed that this information is a measure of the intestinal absorption of the ester. For the purpose of this investigation it was found necessary to devise the analytical method which follows.

Estimation of Phthalates in Urine

While the fluorescein reaction provides a convenient method for the estimation of phthalate ester vapors in air (8), its application to urine is rendered impracticable by a variety of interfering materials. Earlier methods for the determination of phthalic acid in urine depended upon the isolation of the pure compound (4, 5). More recently, however, the general problem of phthalic acid determination has been of interest in connection with the analysis of alkyd resins, and the methods devised have been principally gravimetric, with a variety of weighing forms employed. Of the latter, we have found the lead salt to be adequate after conversion to lead sulfate.

A preliminary separation of phthalic acid from urine is most readily accomplished by ether extraction. Before such an extraction is feasible, however, some pretreatment of the urine is required in order to prevent the formation of stable emulsions. The procedure described by Folin and Flanders (9) eliminates this difficulty without causing any destruction of phthalic acid. Following this preliminary separation the phthalic acid is precipitated as the lead salt, which is then converted to lead sulfate and weighed after ignition. This latter portion of the determination follows the method outlined by Thames (10). The complete procedure is as follows:

Pipette one hundred ml. of urine (50 ml. if concentrated) into a porcelain evaporating dish together with 5 ml. of 10 per cent sodium hydroxide, and evaporate the mixture to dryness on a steam bath. Transfer the residue to a 300 ml. Kjeldahl flask by means of 25 ml. of water and 25 ml. of concentrated nitric acid. Add 0.2 gm. of copper nitrate, a few glass beads, and reflux gently approximately four hours over a micro burner.

After cooling, transfer the contents of the flask to a 500 ml. separatory funnel by the use of about 50 ml. more of water. The total volume of the solution is now about 100 ml. Add to the solution sufficient ammonium sulfate to saturate it (ca. 55 gm.). Make two extractions with 25 ml. each of washed chloroform. These may be discarded, as they serve only to remove benzoic acid and a certain amount of coloring material.

Extract phthalic acid with four successive 50 ml. portions of alcohol-free ethyl ether. The separatory funnel may be shaken vigorously as there is little tendency to form an emulsion. Combine the successive portions of ether in another separatory funnel, and wash once with 50 ml. of saturated sodium chloride. Following this washing, remove the phthalic acid from the ether by extraction with

sodium hydroxide solution. It is essential that an excess of alkali be present in this step; in general, 10 ml. to 15 ml. of a 10 per cent solution will suffice.

Draw off the alkaline phase into a 100-ml. Kjeldahl flask, together with 15 ml. to 20 ml. of wash water and concentrate the entire solution to ca. 15 ml. by boiling. During this process of boiling down, add three portions of 2 ml. each of 3 per cent hydrogen peroxide at intervals, taking precautions to avoid excessive foaming. When the solution has been concentrated to small volume, acidify it with a minimum amount of 20 N sulfuric acid. Bring the acid solution to near boiling, and, while hot, treat it with saturated potassium permanganate solution added dropwise until the last drop is just insufficient to maintain a persistent pink color. This step insures the destruction of oxalic acid which otherwise would interfere in the lead precipitation.

Transfer the acidified solution quantitatively to the central tube of a small continuous extractor of the Clausen type and extract continuously with ether for three hours. To the flask containing the ether extract, add 25 ml. of distilled water, and boil off the ether on a water bath. To the aqueous extract, add a drop of phenolphthalein and bring to neutrality with 10 per cent sodium hydroxide, adding one drop in excess. Precipitate the phthalic acid from this solution by the addition of 10 ml. of 10 per cent lead acetate solution containing 10 ml. of acetic acid per 100 ml. of solution. Allow to stand overnight, and filter using Whatman No. 42 paper and washing three or four times with cold water from the flask onto the filter. The volume of washings should be kept at a minimum. In the event that lead phthalate crystals adhere to the walls of the flask, do not attempt to remove them but allow them to remain, as they have been washed free of lead acetate. Add 10 ml. of 1 to 1 nitric acid to the flask, dissolving these crystals. Now place a 250-ml. beaker beneath the funnel containing the lead phthalate, and wash the nitric acid solution from the flask onto the filter paper. Add several additional 5 ml. portions of 1 to 1 nitric acid to the filter in order to complete washing of the lead phthalate into the beaker. Then add 3 ml. of sulfuric acid, precipitating lead sulfate. Evaporate the nitric acid completely, and take up in 100 ml. of one part alcohol and one part water. Allow to stand 2 hours, filter through a Gooch crucible, ignite in a muffle furnace, and weigh the lead sulfate. The amount of di(2-ethylhexyl) phthalate represented by the lead sulfate is calculated by multiplying the weight of the precipitate by the factor 1.2878.

It has recently been pointed out (11) that lead is unsatisfactory as a precipitant for phthalate in aqueous solutions since the optimum pH value at which phthalate should be determined overlaps the pH value at which lead hydroxide forms. Experiments performed in the course of the present study in which known amounts of potassium acid phthalate were added to control samples of rabbit urine showed that in the range of 50 to 75 mg. of phthalic acid recoveries by the above method were 2 per cent to 5 per cent high, and with quantities of 100 mgm. the positive error was approximately 10 per cent. Despite this uncertainty, the method was considered sufficiently accurate for biochemical use.

Results

Humans. Two adult male subjects swallowed single doses of 10 gm. and 5 gm. respectively of di(2-ethylhexyl) phthalate. In the former case this ingestion was accompanied by mild gastric disturbances and moderate catharsis, while in the latter instance no symptoms whatsoever were noted. In each instance, phthalate equivalent to approximately 4.5 per cent of the dose was recovered from the urine in the succeeding 24 hours. The greater portion was excreted between 5 and 7 hours after the dose.

Dogs. Two male dogs weighing 8.22 kgm. and 10.56 kgm. received single oral doses of 2 gm./kg. of the ester, and daily urine collections were made for three subsequent days. In this period of time, the larger animal excreted phthalate equivalent to 4.5 per cent of the dose, while 2.0 per cent was accounted for in the case of the smaller. In each case, the greatest amount was excreted during the second day after dosing.

Rabbits. The urinary excretion of phthalate was studied in five adult male albino rabbits (2 to 3 kg. in weight) following single oral doses of 2 gm./kg. of di(2-ethylhexyl) phthalate. Urine collections were made for three successive days after dosing, since experience showed that considerable amounts of phthalate were excreted even on the last day of this period. The largest part of the dose accounted for in this time was 65.4 per cent in one case, while in another phthalate equivalent to only 26 per cent of that administered was found. The remaining three animals excreted phthalate equivalent to 33.8, 43.8, and 40.5 per cent of the dose respectively.

Rats. No quantitative data were gathered on this species, but considerable amounts of phthalic acid were recovered from the urine of rats fed the ester. It is probable, therefore, that the rat is similar to the rabbit in its inability to metabolize this acid.

Discussion

Because study of the metabolism of *di*(2-ethylhexyl) phthalate has revealed that the ester is saponified in the body, o-phthalic acid being excreted unchanged and none of the ester appearing in the urine, we have estimated the LD_{50} of the alcohol and acid where administered separately to rats. It is true that Hodge (1) has reported the oral LD_{50} of 2-ethylhexanol to be 3.2 gm./kg. for rats, but he used heavy animals of mixed sex and unspecified strain. With many materials we find these factors cause significant differences in the LD_{50} and therefore decided to repeat his work, using our 90 to 120 gram male Wistar strain white rats.

Phthalic acid was administered as a solution of the mixed sodium and potassium salt adjusted to pH 7, 1 ml. equivalent to 0.50 gm. o-phthalic acid. The 2-ethylhexanol was dispersed in 1 per cent "Tergitol" penetrant 7; 1 ml. containing 0.20 gm. The results are listed below.

o-phthalic acid, LD_{50}, 7.9 (7.5 to 8.4) gm./kg.
Slope 16.11 ± 7.80

2-ethylhexanol, LD_{50}, 7.1 (5.5 to 9.1) gm./kg.
Slope 4.09 ± 2.64

di(2-ethylhexyl) phthalate, LD_{50}, 30.6 (20.8 to 45.2) gm./kg. Slope 1.92 ± 0.98

It is evident that the slopes of the dosage-mortality lines of the alcohol and ester are not significantly different while that of the acid is widely different from the former two. This fact is an indication that the mechanism of action of the ester is similar to that of the alcohol rather than to that of the acid. Further indirect evidence for difference of action lies in the fact that o-phthalic acid could be recovered from the urine of dosed rats and rabbits while more than half of the fatty acid recovered was of lower molecular weight than 2-ethylhexoic acid.

The LD_{50} of the ester will produce upon saponification 13.0 gm./kg. of phthalic acid and 20.4 gm./kg. of 2-ethylhexanol. The figures listed above show that if a mixture of the acid and alcohol in these dosages were fed, all of 10 rats would die except in the unlikely case of a strong antagonism between the injurious actions of the two. However, no unchanged ester was found in the urine. Therefore only part of the dose of the ester could have been absorbed from the digestive tract, and in fact phthalate was demonstrated qualitatively in feces.

Approximately 35 per cent is absorbed because the only dosage of a mixture of acid and alcohol in the proportions hydrolysis yields, which will kill 50 per cent of the rats is 7.1 gm./kg. of alcohol and 4.6 gm./kg. of the acid if the two have independent joint toxic actions. If there is some degree of association between the toxic actions, the proportion of ester absorbed would be somewhat more. The average recovery of phthalic acid in the urine of rabbits receiving the ester indicated about 70 per cent absorption, which supports the foregoing argument. A dosage of 4.6 gm./kg. of o-phthalic acid would kill about 0.01 per cent of the rats receiving it, according to the dosage-mortality line. Our data do not justify projecting the line to such low mortalities, but it is nevertheless apparent that o-phthalic acid can have little if any influence upon the toxicity of di(2-ethylhexyl) phthalate. The toxicity is largely or entirely due to the alkyl part of the molecule and it seems likely that even less toxic plasticisers can be produced by esterifying relatively strongly ionized acids like o-phthalic with alcohols of toxicity lower than 2-ethylhexanol. But it must be realized that this argument is based upon experiments with animals which are unable to metabolize o-phthalic acid and that it may not be valid with species which can effect its oxidation. Our human experiments showed a high recovery of phthalic acid from the urine and, if human absorption is equal to that of rats and rabbits, the human does metabolize the acid as do the animals known to do (5).

Summary

Animal experiment of limited scope has shown that di(2-ethylhexyl) phthalate is a chemical of low toxicity and that the health hazards involved in its use as a plasticizer are slight. Such injurious action as it does exert within the body appears to be due to the alkyl part of the molecule rather than to the phthalate portion.

REFERENCES

(1) Hodge, H. C.: Acute toxicity for rats and mice of 2-ethylhexanol and 2-ethylhexyl phthalate. Proc. Soc. Exper. Biol. & Med., *53:* 20–23, 1943.

(2) Bliss, C. I.: The determination of the dosage-mortality curve from small numbers. Quart. J. Pharm. & Pharmacol., *11:* 192–216, 1938.

(3) Calvery, H. O.: Safe guarding foods and drugs in wartime. American Scientist, *32:* 103–119, 1944, and personal communication.

(4) Juvalta, N.: Ist der Benzolkern im Thierkörper zerstörbar. Z. physiol. chem., *13:* 26–31, 1889.

(5) Pribram, E.: Zur Lehre von den physiologischen Wirkungen carbocyclischer Säuren. Arch. f. Path u. Pharm., *51:* 372–382, 1903.

(6) Quick, A. J.: The relationship between chemical structure and physiological response. J. Biol. Chem., *97:* 403–419, 1932.

(7) Friedemann, R. E.: The identification and quantitative determination of volatile alcohols and acids. J. Biol. Chem., *123:* 161–183, 1938.

(8) Jacobs, M. B.: Analytical chemistry of industrial poisons, hazards, and solvents. New York, 518–519, 1941.

(9) Folin, O., and Flanders, F. F.: A new method for the determination of hippuric acid in urine. J. Biol. Chem., *11:* 257–263, 1912.

(10) Thames, F. C.: Determination of phthalate plasticizers. Ind. Eng. Chem., Anal. Ed., *8:* 418–419, 1936.

(11) Smith, S. B., and Stremmfer, J. F.: Determination of phthalate. Ind. Eng. Chem., Anal. Ed., *16:* 416, 1944.