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The Effect of Feeding Di-(2-ethylhexyl) Phthalate (DEHP) on the Lipid Metabolism of Laying Hens

1/1/80 Rat

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ABSTRACT

Di-(2-ethylhexyl)phthalate (DEHP), a commonly used plasticizer, is now seen as an environmental pollutant. DEHP has been found to inhibit lipid and sterol synthesis in rats and mice. The effects of DEHP on various aspects of lipid metabolism were examined in chickens. White Leghorn laying hens were fed either a standard laying mash control diet (C) or the control diet containing 1% DEHP (DEHP) or 1% DEHP and 5% tallow (DEHP-T) for 28 days. DEHP and DEHP-T lowered feed consumption 10% but did not significantly affect body weight. After 3 weeks on the diets, egg production was 15-20% less in DEHP-T than in C and DEHP hens. No differences were observed in egg weight, percent shell, white or yolk among the groups. DEHP and DEHP-T did not alter egg lipid or egg cholesterol concentrations. DEHP and DEHP-T lowered plasma lipid concentration about 20% and free and total cholesterol 20-30%. Liver weights increased, being 30, 34 and 39 g for C, DEHP and DEHP-T hens, respectively, after 28 days. Total liver lipid and cholesterol increased 19% and 26% in DEHP hens and 54% and 79% in DEHP-T hens when compared to controls. In contrast, the fat content of pectoralis major muscle decreased significantly in DEHP and DEHP-T hens. These results, in showing that DEHP alters plasma and tissue cholesterol but not yolk cholesterol, demonstrate again that egg cholesterol is remarkably resistant to alteration by dietary means.

INTRODUCTION

Di-(2-ethylhexyl)phthalate (DEHP), a phthalic acid ester commonly used as a plasticizer in the production of polyvinyl chloride, is now considered an environmental pollutant (1). Since the phthalic acid esters are not chemically bound to the plastics, they can migrate from the plastics to the environment (2) and into man (3) and animals (4,5). The wide distribution in the environment, and the annual production of nearly one billion pounds of phthalic acid esters in the United States (6), has led to many studies on the toxicological and biological effects.

Recent studies have shown that DEHP affects lipid metabolism in the rat, decreasing acetate incorporation into liver lipids (7) and sterols (8) in vitro. DEHP also exerts hypolipidemic effects in rats and mice (9). These studies were done to examine the effects of dietary DEHP on lipid metabolism in the hen, and more specifically, to determine whether DEHP would lower egg yolk cholesterol. Control laying hens were compared to hens fed DEHP and to a group of hens fed DEHP and tallow. Tallow was added to insure that enough lipid was available for egg production in the event DEHP exerted severe lipid lowering effects.

EXPERIMENTAL PROCEDURES

Animals and Diets

Single-comb White Leghorn pullets were obtained from a commercial hatchery at 20 wk of age and caged individually. The birds were given a 14-hr light regimen daily and a standard breeder mash ration. When the birds reached 70% egg production, they were distributed into 3 groups of 15 each on the basis of body weight. The mean initial body weights were 1654, 1684, and 1721 g for the control (C), DEHP (DEHP) and DEHP plus tallow (DEHP-T) groups, respectively.

The control group was fed ad libitum the standard laying mash for 6 wk (1 wk pre-experimental, 4 wk experimental and 1 wk post-experimental periods). During the 4 wk experimental period, the DEHP group was fed ad libitum the standard laying mash containing 1% DEHP and the DEHP-T group was fed ad libitum the standard laying mash containing 1% DEHP and 5% tallow. During the period from days 28-35, all groups were fed the standard laying mash ad libitum. The fat content of the diets was determined gravimetrically after ethyl ether extraction using a Goldfisch fat extraction apparatus.

The control diet was a standard laying mash that contained 63.2% corn meal, 19.0% soybean meal, 5.0% alfalfa meal, 2.0% meat and bone, 7.0% limestone, 2.5% dicalcium phosphate, and 1.3% salts-vitamins-minerals. The diets containing 1% DEHP or 1% DEHP plus 5% tallow were prepared just prior to the start of the experiment by adding the appropriate amount to the laying mash and mixing. The DEHP was obtained from Eastman Kodak Co., Rochester, NY, and the tallow was stabilized white tallow. The diets were stored in a 4 C room until used. The control and DEHP diets contained 16.1% protein, 3.5% calcium, and

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2727 kcal/kg metabolizable energy. The diet containing DEHP and tallow had 15.2% protein, 3.3% calcium and 2943 kcal/kg metabolizable energy. The control diet, the DEHP diet and the DEHP-T diet were 90.9, 90.6 and 88.4% dry matter, respectively. The control and DEHP diets contained 5.0% fat and the DEHP-T diet contained 9.7% fat, on a wet weight basis.

Experimental Protocol

The variables studied were: feed consumption, body weight changes, egg production, egg weight, egg composition (percent shell, white and yolk), yolk fat, total yolk cholesterol, free yolk cholesterol, plasma cholesterol (free and total), liver composition, liver lipids and cholesterol.

Blood was collected from the brachial vein of 4 hens from each group on days 0, 7, 14 and 21. Brachial vein blood withdrawal from White Leghorn hens is difficult and frequently causes substantial hematomas, consequently, the birds could not be bled from that wing vein at the succeeding time period. Plasma lipid and cholesterol data thus are not from the same hens at all time periods. The difficulty in bleeding dictated an observation number of 4 for the experimental variables at 0, 7, 14 and 21 days. At 28 days, all hens were bled and 4 hens from each group were randomly selected and killed to obtain livers and pectoralis major muscle for analysis.

The following orthogonal contrasts were studied: C vs DEHP + DEHP-T, DEHP vs DEHP-T. Analysis of covariance for heterogeneity of slopes was used to examine egg production, feed consumption and body weight data (10). The rest of the data was subjected to a one-way analysis of variance based on the a priori contrasts.

Egg Yolk Analyses

Eggs collected daily were stored at 13 C and 80% humidity. Within 4 days of collection, eggs were hard boiled, weighed and percentage of shell, white and yolk determined gravimetrically. Yolk was stored at -20 C until analyzed.

Petroleum ether-ethanol was found to extract egg yolk cholesterol satisfactorily. A weighed sample of yolk (0.8 g) was homogenized in a 50 ml teflon-lined, screw-capped test tube with 18 ml petroleum ether-ethanol (2:1) using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). After 6 ml of distilled water were added, the tubes were shaken by hand, phases separated by centrifuging 10 min at 260 x g, and a 1.0 ml aliquot of the petroleum ether phase was transferred to a small vial for gas liquid chromatographic analysis (GLC) of free cholesterol. After evaporation of the petroleum ether, 1.0 ml of a 20% solution of TRI-SIL (mixture of hexamethyldisilazane and trimethylchlorosilane, Pierce Chemical Company, Rockford, IL) in pyridine, containing 0.5 mg cholestane as an internal standard, was added to each vial. The GLC vial was capped and heated at 75 C for 30 min to form the trimethylsilyl (TMS) derivatives. The GLC analyses of cholesterol were made on a 6 ft glass column containing 3% OV-17 coated on GAS-CHROM Q (100/120 mesh) with a Model 7620 Hewlett Packard gas chromatograph (column temp.: 250 C, He flow rate: 30 ml/min).

Percent yolk lipids were determined by transferring a 4.0 ml aliquot of the petroleum ether phase to a weighed 50 ml screw-capped test tube, then by evaporating the solvent and weighing the residue. Total yolk cholesterol was determined by adding 0.3 ml distilled water and 6.0 ml of 2.5% KOH in ethanol to the dried residue and heating at 70 C in a shaking water bath for 90 min to saponify cholesteryl esters. Cholesterol was extracted by adding 12 ml petroleum ether and 6 ml distilled water, shaking and phasing by centrifugation. A 1.0 ml aliquot of the petroleum ether phase was taken for TMS-derivatization with GLC analysis as described for free cholesterol.

Analysis of Liver, Muscle and Blood

Samples of liver, muscle and plasma were frozen, later homogenized in petroleum ether-ethanol and the lipids extracted by the procedure outlined above for egg yolk lipids. Lipids were determined gravimetrically on dried aliquots of the extracts. Total and free cholesterol also were determined as TMS derivatives by GLC analysis as described for yolk cholesterol.

RESULTS AND DISCUSSION

Data for feed consumption and body weight are presented in Figure 1 and Table I. DEHP and DEHP-T caused a decrease in intake which was observed within the first wk (Fig. 1). Feed consumption for the entire 4-wk experimental period was 10% lower, being 102 for control but only 93 and 91 g/hen/day for DEHP and DEHP-T hens. During the second half of the experimental period, the DEHP hens appeared to adapt to the DEHP diet and feed consumption approached control intake. The DEHP-T hens showd a greater decline in feed consumption during the last week of the experimental period but recovered somewhat during the post-experimental period. These depressions

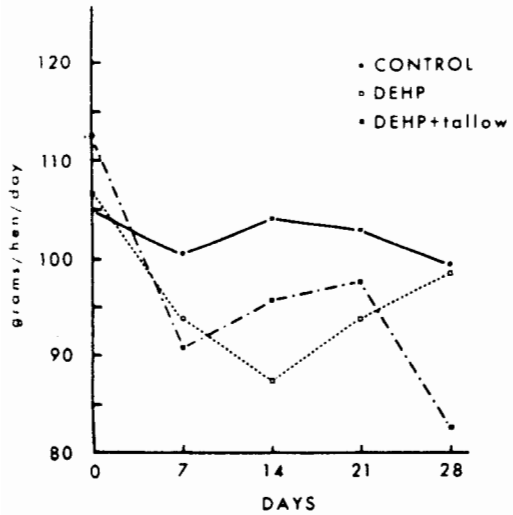

FIG. 1. Feed consumption of hens fed control, DEHP or DEHP + tallow diets for 28 days.

about 10% in feed consumption were significant at $P < .05$ (control vs DEHP and DEHP-T). No overt signs of debilitation were observed in any group. The 10% decrease in feed consumption was not reflected to the same degree in body weight changes. Consistent losses in body weight of about 2-3% were observed in the DEHP and DEHP-T hens (Table I), but these variations were not statistically significant differences.

During the third wk of feeding DEHP + tallow, egg production was 20% less than controls (Table II). Egg production for the 4-wk experimental period was 14% lower in DEHP-T, and 5% lower in DEHP, than in control hens. No differences, however, were observed in egg weight, percent shell, white or yolk among the groups (control egg data: 57.0 g egg weight, 11.0% shell, 57.2% white and 31.8% yolk). Yolk lipid and cholesterol of eggs from DEHP and DEHP-T hens were not different from controls (% lipid, 33.8; total cholesterol, 14.58 mg/g yolk; % free cholesterol, 91.0).

The effects of feeding DEHP or DEHP + tallow on plasma lipid and cholesterol concentrations, expressed as percent of control values are shown graphically in Figure 2. Because of small sample size (n = 4), levels during the pre-experimental period (0 wk) were higher than controls. Plasma lipids were reduced about 20% but the variations were not statistically different. Plasma total and free cholesterol were lowered about 20% relative to controls ($P < 0.01$). Free cholesterol remained about 77% of total cholesterol in C, DEHP and DEHP-T plasma.

Liver weights of the hens fed DEHP or DEHP + tallow increased (Table III) but the differences were not statistically significant. Liver composition, fat and water were not different in DEHP and DEHP-T groups compared to controls. Total liver lipid increased 19% in DEHP and 54% in DEHP-T hens ($P < 0.01$). The concentration of cholesterol and cholesteryl ester increased in the DEHP and DEHP-T livers ($P < 0.04$). Because of the accompanying increase in liver size, total cholesterol increased 29% in DEHP and 81% in DEHP-T livers.

Cholesterol and lipid were also measured in the pectoralis major muscle (Table IV). Total cholesterol and free cholesterol concentrations increased ($P < 0.05$) but fat content decreased significantly in both DEHP and DEHP-T muscle ($P < 0.05$).

Our study has demonstrated that feeding 1% of the plasticizer DEHP in the diet modified some aspects of lipid metabolism in laying hens. Body weight did not significantly change but feed consumption and egg production were lowered. The decreased feed consumption in the DEHP-T hens may have been the result of the increased caloric content of the diet. However, the caloric content of the DEHP feed was similar to the

TABLE I

Body Weight Changes of White Leghorn Hens Fed DEHP

Treatment	Initial body weight[a] (g ± SE)	Change from initial body weight (g) Days of experiment				
		7	14	21	28	35
Control	1654 ± 30	+18	-3	+1	+7	+33
DEHP	1684 ± 26	-99	-25	-21	-39	-25
DEHP-T	1721 ± 24	+6	-30	-56	-85	-60

[a]Values are means ± SEM for 15 White Leghorn hens in each group.

TABLE II

Effect of DEHP and Tallow Feeding on Egg Production[a]

Treatment	% Egg production during week: 1	2	3	4	5	% prod 0-28
Control	80	75	80	81	75	
DEHP	74	69	70	77	81	
DEHP-T	79	72	60	61	66	
C vs DEHP and DEHP-T	NS	NS	< .01	< .01	NS	
DEHP vs DEHP-T	NS	NS	< .01	< .01	NS	
Standard error of the mean	4	4	3	3	4	

[a]Egg production was calculated weekly on a percentage basis, 100% being 1 egg/hen/day; each grou sisted of 15 hens.

control diet and a depression in feed consumption was still apparent. The decreased egg production in both groups may have resulted either from their decreased feed consumption or from a physiological effect of DEHP. Liver weights increased but this trend was not statistically significant. In contrast, rats fed DEHP usually show a marked hepatomegaly (7, 11-14).

In our study, plasma lipids and cholesterol showed a 20% decrease after feeding 1% DEHP for 1 wk, effects similar to those observed in rats and mice (9,11,14). However, much greater effects were observed on lipid and cholesterol levels in the chicken liver: total lipid and total cholesterol content increased about 70%. Plasma cholesterol decreased in DEHP-fed rats

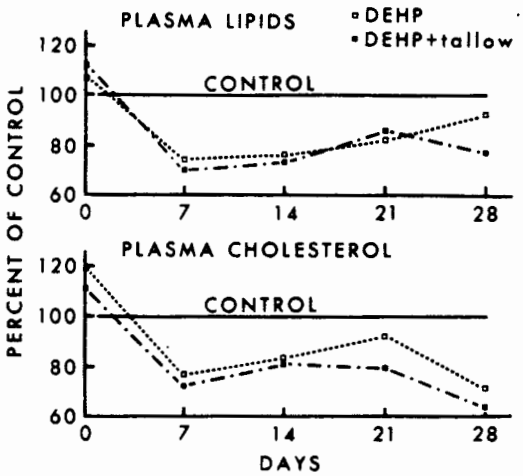

FIG. 2. Plasma lipids and cholesterol of hens fed control, DEHP or DEHP + tallow diets for 28 days. The overall means (± SE) of control plasma concentrations were: total lipids 2382 ± 144 mg/100 ml; cholesterol 127 ± 6 mg/100 ml. n at 0, 7, 14, 21 days: 4; n at day 28:15.

and rabbits without an accompanying inc in liver cholesterol concentration (11 Studies in rats, however, generally sho creases in the total lipid and cholesterol co of the liver of about this magnitude placed on a whole organ basis (11,14,16). our results confirm that DEHP affects metabolism in an avian species in a ma similar to that reported for mammalian sp

The mechanism by which cholesterol lo plasma lipids and cholesterol has not elucidated. The presence of desmosterol not observed in the plasma, liver or m samples of the hens in this experiment, in ing the cholesterol and lipid reduction di take place by inhibition of the Δ^{24}-s reductase step in the conversion of desmos to cholesterol (17).

Several hypotheses have been propose the hypolipidemic mechanism of actio DEHP: (a) increased lipid oxidation cause DEHP- induced peroxisome proliferation decreased lipid biosynthesis; (c) inhibitio energy-linked reactions, and (d) feed control of cholesterol on its own biosynt

Peroxisome Proliferation

Liver peroxisome proliferation is ch teristic of hypolipidemic compounds an been demonstrated for many unrelated lowering agents (18-22). Several cata lipid-related enzymes have been shown associated with liver peroxisomes, incl catalase (23), carnitine acetyl transferase and recently, the β-oxidative enzymes (25 increase in liver catalase and carnitine transferase activity was found when DEH fed to rodents (9,13,26). In addition, β-oxidation increased significantly in administered DEHP for 1 wk (26). Ther increased activities of catabolic lipid en associated with liver peroxisomes cou

responsible for DEHP's plasma lipid lowering effects.

Decreased Lipid Synthesis

A consistent decrease in labeled acetate and mevalonic acid incorporation into various lipid fractions was found in both in vitro and in vivo experiments with rats (7-8,11,27-28). Decreased incorporation of label into squalene and sterols was observed and significant reductions in fatty acyl lipids were also noted, implying inhibition of an enzyme common to both groups. A decrease in acetate activation has been suggested as a possibility (11), since that enzyme would be required for de novo synthesis of cholesterol and fatty acyl lipids. A reduction in acetate incorporation before an effect upon mevalonic acid incorporation into rat liver squalene and sterols suggested a possible site for DEHP's inhibitory effect on liver cholesterol biosynthesis would be at the enzyme 3-hydroxy-3-methyl-glutaryl CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis (11).

Inhibition of Energy Linked Reactions

A reduction in liver electron transport enzyme activities and inhibition of succinic dehydrogenase and adenosine triphosphatase in heart, liver, kidney, testes and ovaries of DEHP-treated rats have been found (15). In addition, an inhibition of liver mitochondria respiration and uncoupling of oxidative phosphorylation by phthalate esters (29-30) has led to a conclusion that the suppression of energy-linked reactions may be a generalized DEHP effect on cholesterol content (16).

Feedback Control

The accumulation of liver cholesterol could inhibit 3-hydroxy-3-methyl-glutaryl CoA reductase, reducing liver synthesis of cholesterol (31). Accumulation of liver cholesterol on a total liver basis was found in hens fed DEHP. It is possible this resulted in decreased liver cholesterol biosynthesis which reduced circulating cholesterol.

Egg lipids and cholesterol were unaffected in spite of a 20% reduction in plasma cholesterol, demonstrating that egg cholesterol is resistant to alteration by dietary agents (32,33). This may reflect the removal of a negative feedback inhibition upon ovarian cholesterol biosynthesis. As plasma cholesterol falls, the feedback inhibition on ovarian cholesterol biosynthesis may be removed, resulting in normal levels of yolk cholesterol, even though plasma cholesterol has been depressed. Signifi-

TABLE III

Effect of Feeding DEHP or DEHP + Tallow in Hepatic Parameters of the Hen[a]

	Liver weight g	% of body weight	% H_2O	% Fat	Total lipid g	Cholesterol mg/g DW	Cholesteryl ester mg/g DW	Total cholesterol mg	Total cholesteryl ester mg
Control	29.6	1.84	69.6	17.3	5.1	7.8	0.8	68.5	6.7
DEHP	34.1	2.07	70.4	17.8	6.1	8.7	1.4	88.1	13.5
DEHP + Tallow	38.8	2.29	68.7	20.3	8.1	10.2	2.0	123.7	24.5
Standard error of the mean	2.8	0.2	1.1	1.8	1.2	0.6	0.2	9.8	2.8
C vs DEHP and DEHP-T	NS	NS	NS	NS	NS	<.04	<.02	<.01	<.01
DEHP vs DEHP-T	NS	NS	NS	NS	NS	NS	NS	<.01	<.01

[a]Values are the means of 4 hens.

TABLE IV

Effect of Feeding DEHP or DEHP + Tallow on Cholesterol and Lipid Content of the Pectoralis Major Muscle[a]

Group	Total cholesterol mg/100 g WW	Free cholesterol mg/100 g WW	Lipid %
Control	60.9	55.9	4.3
DEHP	67.1	57.8	3.6
DEHP + Tallow	73.7	65.7	1.6
Standard error of the mean	3.4	2.7	0.6
C vs DEHP and DEHP-T	P<0.05	NS	P<0.05
DEHP vs DEHP-T	NS	P<0.05	P<0.05

[a]Values are the means of 4 hens.

cant in vitro ovarian cholesterol synthesis has been reported (34). However, in vivo experiments indicate that little, if any, ovarian synthesis occurs under normal conditions (35).

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