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Concentration, Distribution, and Withdrawal of
Ethylene Dibromide (EDB)
in Eggs and Tissues Obtained from Chickens
Fed Diet Containing EDB-Contaminated Flour

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CONCENTRATION, DISTRIBUTION, AND WITHDRAWAL OF ETHYLENE DIBROMIDE (EDB) IN EGGS AND TISSUES OBTAINED FROM CHICKENS FED DIET CONTAINING EDB-CONTAMINATED FLOUR

by

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ABSTRACT

CONCENTRATION, DISTRIBUTION, AND WITHDRAWAL OF ETHYLENE DIBROMIDE (EDB) IN EGGS AND TISSUES OBTAINED FROM CHICKENS FED DIET CONTAINING EDB-CONTAMINATED FLOUR

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Hens were fed diet containing 6.7 ppm EDB for 21 days followed by 21 days of non-contaminated diet (days 0 to 21 of withdrawal). EDB residues in egg, whole body, fat, muscle, liver, kidney, and skin were quantified with head space GC methodology. Tissues and eggs contained less than one percent of EDB intake. Eggs contained detectable EDB by day 3 of feeding contaminated diet and reached a plateau of 28 ppb by day 8. By day 6 of withdrawal EDB was not detectable in eggs. Concentration of EDB on day 0 of withdrawal in whole body, fat, and muscle was 11, 54, and 0.44 ppb, respectively. Fat contained 95% of whole body residues. EDB was not detected in liver, kidney, and skin on day 0 of withdrawal and was not detected in tissues on day 21 of withdrawal. Activities of hepatic mixed function oxidases were not induced by feeding EDB at 6.7 ppm for 21 days.

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INTRODUCTION

In 1938 a food law was passed that prohibited sale of insect infested grains. This prompted the development of several grain insecticides, one of which was the fumigant ethylene dibromide (1,2-dibromoethane or EDB), an aliphatic halogenated hydrocarbon. EDB was first registered for use as a grain fumigant in 1948. It was found to be an effective method for control of insect infestations in stored grains (Girish et al. 1972). However, problems with its use were encountered in 1958 when several poultrymen in South Carolina reported substantial reductions in egg size and egg production after feeding their flocks oats that had been fumigated with an EDB fumigant (Caylor and Laurent 1960). Restrictions were not set against use of EDB as a grain fumigant because: 1) at that time there was no indication that EDB was toxic to humans and 2) it was assumed that proper processing would eliminate EDB residues from grains (Environmental Protection Agency 1977). Use of EDB as a grain fumigant was continued until February 3, 1984 when William D. Ruckelshaus, Administrator of the Environmental Protection Agency (EPA), issued an emergency order suspending registration of EDB pesticides used to fumigate grain milling equipment and stored grains. Mr. Ruckelshaus issued this order because of evidence that consumption of EDB-contaminated grain "posed increased risk of cancer, heritable genetic damage, and

adverse reproductive effects" to the general public (EPA 1984). On April 23, 1984, the EPA established tolerance limits for EDB *per se* in grain as follows: 1) 900 ppb in non-processed grains, 2) 150 ppb in milled products such as flour, and 3) 30 ppb in finished ready-to-eat products such as cereals. Any grain products that contain more EDB than what is specified by the tolerance limits cannot be marketed (Food and Drug Administration 1984).

On February 2, 1984, the Michigan State Department of Agriculture confiscated 50 pounds of EDB-contaminated flour from the Amendt Milling Company of Monroe, Michigan. The flour contained 31.1 ppm EDB and was made available to ascertain if chickens fed this food item would deposit EDB into eggs and tissue and thus, present a source of EDB contamination to humans. The objectives of this study were:

1) To develop an assay with detection limits by which residues of EDB in tissues and eggs could be quantified.

2) To quantify residues of EDB in tissues and eggs obtained from hens fed diet containing EDB-contaminated flour.

3) To determine if subsequent withdrawal of EDB-contaminated diet from hens would reduce EDB residues in tissues and eggs.

4) To determine if dietary exposure to EDB would induce hepatic mixed function oxidase activity in chickens.

LITERATURE REVIEW

I. Chemical Properties of EDB

Ethylene dibromide (1,2-dibromoethane, symdibromoethane, glycol bromide, or EDB) is an aliphatic halogenated hydrocarbon produced commercially by reacting gaseous ethylene with liquid bromine. EDB is known commercially as Bromofume, Orthofume, Dowfume W-85, Escobrome-D, Bromotox, Celmide, Nephis, or Kop Fume. Its chemical formula is $Br-CH_2-CH_2-Br$, and its molecular weight is 187.86. It is a heavy, colorless non-flammable liquid at room temperature which turns brown when exposed to light. It has a chloroform-like odor detectable in air by humans at a concentration from 10 to 25 ppm (77 mg/m^3 to 192.5 mg/m^3). EDB is readily soluble in most organic solvents and is slightly soluble in water (0.43 g/100 g at 30°C). EDB also has the following properties:

Specific gravity	$= 2.172^{25}_{25}$
Melting point	= 9.6°C
Boiling point	= 131.4°C at 760 mm Hg
Density	= 2.18 g/ml at 20°C
Vapor pressure	= 11 mm Hg at 25°C
Vapor density	= 6.5 (air = 1.0)
Viscosity	= 1.65 centipoise at 20°C
Heat of vaporization	= +53 cal/g at 25°C with no flash point

The preceding information was taken from Girish *et al.* (1972), EPA (1977), and the Department (Dept.) of Labor (1983).

II. Production and Uses of EDB

A. Production and uses prior to banning of EDB as a pesticide

The EPA estimated that prior to bans against use of EDB as a pesticide approximately 135,000 to 160,000 metric tons of EDB were produced annually in the United States by PPG Industries, Ethyl Corporation, Great Lakes Chemical Corporation, Dow Chemical Corporation, and Velsicol Chemical Corporation. About 30% of the EDB produced was exported, 50 to 60% was used as a pesticide, and a small amount was used as an intermediate in the synthesis of dyes and pharmaceuticals and as a solvent for resins, gums, and waxes (Brown 1984, EPA 1977, Dept. of Labor 1983).

Pesticide uses of EDB have included: 1) soil nematocides (85 to 90%), 2) fumigation of stored grain and grain milling equipment (6 to 10%), 3) fumigation of citrus fruit (1 to 2%), and 4) control of pinebark beetles, termites, and wax moths in honey combs (1%). In 1983, 160 million bushels of wheat, 14 million bushels of corn, and 30 million bushels of other grains (oats, barley, rice, rye, and sorghum) were fumigated with EDB alone or in admixture with one or more of the following chemicals: carbon tetrachloride (CT), ethylene dichloride (EDC), methyl bromide (MB), chloroform, carbon disulfide (CD), sulfur dioxide (SD), or benzene. Some typical combinations were: CT:EDC:EDB (Dowfume EB-5, 63:30:7 w/w), EDB:MB (70:30 w/w), and CT:CD:SD:EDB:pentane (80.9:16.1:1.5:1.2:0.4 w/w). The application rate of EDB varies with the facility or grain being fumigated but usually ranges from 0.7 to 1.4 kg per 30 cubic meters for spot milling or 0.9

to 1.8 kg per 1000 bushels of grain with reapplication at the first sign of reinfestation (Brown 1984, EPA 1977; EPA 1984, Dept. of Labor 1983, Gilby 1983, Girish et al. 1972).

Pesticidal use of EDB has been for control and not prevention of insect infestations. EDB is applied as a liquid which then volatilizes, and as such is absorbed thorugh the insect's respiratory system. It acts as an asphyxiant by interfering with enzymatic functions associated with cell respiration (Christensen 1974).

B. Bans, current uses, tolerance limits, and alternatives to EDB

On December 14, 1977, the EPA issued a rebuttable presumption against registration (RPAR) and continued registration of pesticide products containing EDB. Issuance of a RPAR is the first step towards suspending use of a pesticide. It is sisued when evidence exists that a pesticide meets or exceeds risk criteria relating to acute and chronic toxic effects outlined in the Federal Insecticide, Rodenticide, and Fungicide Act. In the case of EDB, a RPAR was issued because of evidence that 1) EDB is sorbed by food during fumigation and 2) prolonged consumption of EDB-contaminated food could cause cancer or interfere with reproduction. Thus, in 1984, all but the following uses of EDB as a pesticide were cancelled: 1) fumigation of exported fruit and 2) control of wax moths, beetles, and termites. Non-pesticidal uses of EDB have not been cancelled. Tolerance limits of EDB in raw grain, milled grain, ready-to-eat grain products, whole fruit, edible pulp of fruit, and ready-to-eat honey are 900, 150, 30, 250, 30, and 30 ppb, respectively.

Alternatives to pesticidal use of EDB include: 1) the liquid

fumigants CT, CD, SD, EDC, and chloropicrin, 2) the solid fumigants aluminum and magnesium phosphide, 3) the gaseous fumigant methyl bromide, 4) cold treatment, and 5) irradiation. As of July 1, 1986 use of CT, CD, SD, and EDC will be cancelled. Use of methyl bromide is likely to be cancelled in the near future also. Irradiation is a promising alternative, but grain and citrus producers have been unwilling to adapt their storage areas to accomodate its use. Thus, as of now, insect infestations in mills, grain, and fruit are being controlled with cold treatment and the fumigants chloropicrin and aluminum or magnesium phosphide (Tangley 1984 and EPA 1985).

III. Sorption and Residues of Ethylene Dibromide in Grain

Grains chemically and physically sorb EDB. Chemical sorption occurs primarily by the protein fraction of teh grain (endosperm) via alkylation and release of a bromide molecule. Chemical sorption is irreversible and at 25°C accounts for 10-20% of the total amount of EDB sorbed. It has been determined that chemically sorbed EDB and the bromide released are not the cause of toxic effects observed in poultry and mammals. Physical sorption of EDB is reversible, does not chemically alter EDB, involves both absorption and adsorption, accounts for up to 90% of the total amount of EDBV sorbed at 25°C, and is the cause of toxic effects observed in poultry and mammals (Olomucki and Bondi 1955, Bondi et al. 1955, Berck and Gunther 1970, EPA 1977).

The amount of EDB sorbed by grain has been quantified by

several methods. Some researchers have measured the decrease in concentration of EDB over time in an airtight atmosphere following fumigation (Berck 1965, Vincent and Lindgren 1971). EDB lost from the atmosphere was assumed to have been sorbed by the grain. These studies could not distinguish between chemically and physically sorbed EDB, but they did show that the amount of EDB sorbed increases with decreasing temperature, increasing moisture content of the grain, and increasing surface area (decreasing particle size). These studies also showed that up to 55% less EDB is sorbed when it is applied in an admixture than when it is applied singly. Several studies have reported residues of EDB in grain as total bromide content instead of as EDB per se (Girish and Kumar 1975, Girish et al. 1972), but the bromide levels reported did not distinguish between bromide originating from chemically sorbed EDB and bromide bound to physically sorbed EDB . Once it was determined that it is chemically unaltered EDB which is responsible for toxic effects observed in poultry and mammals, researchers began quantifying residues of EDB per se in whole grain and grain products. These studies have consisted of 2 types: 1) whole grain was fumigated with EDB, aerated, processed, and then analyzed for EDB content and 2) random samples of grain products were removed from commercial enterprises and analyzed for presence of EDB.

Berck (1974) quantified residues of EDB per se in wheat and milled wheat products after fumigating whole grain with 28 kg of Dowfume EB-5 per 1000 bushels. Dowfume EB-5 is 7% EDB so this is equivalent to a dose of 2.0 kg of EDB per 1000 bushels. The wheat

contained 14% moisture, was stored in a paper laminate bin, and was fumigated on the third, seventh, fourteenth, twenty-fifth, forty-second, and forty-ninth day of storage. After the last fumigation, concentration of EDB was measured in whole grain and in bran, middlings, flour, and bread derived from the whole grain. Whole grain contained 10 to 1360 ppb EDB, bran and middlings contained 20 to 220 ppb EDB, and flour contained 10 to 20 ppb EDB. Bread samples did not contain detectable EDB (detection limit = 0.05 nanograms). Therefore, after prolonged exposure to EDB some of the samples analyzed contained more EDB than allowed by the current grain tolerance limits (see introduction for grain tolerance limits).

Anderson et al. (1985) quantified residues of EDB per se in whole corn and milled corn products after fumigating whole grain with an admixture of CT, CD, SD, EDB, and pentane (80.9:16.0:1.5:1.2:0.4 w/w). Every 91 kg of corn received 40.5 ml of admixture. This is equivalent to a dose of 1.3 g EDB per 91 kg of corn, or approximately 360 g EDB per 1000 bushels of corn. The corn was exposed to the fumigant for 5 days at 25°C in an air tight steel drum. Residues of EDB were measured at 0, 30, and 180 days post-fumigation in whole grain and in hulls, germ, starch, gluten, flour, bran meal, oil, hominy, masa, and tortillas derived from whole grain. It was found that EDB concentrated in the germ and hull, but all residues detected (detection limit = 1 ppb) were below current grain tolerance limits of EDB.

Several other researchers have measured EDB per se in grain and

milled products after fumigation. Caylor and Laurent (1960) found 10-15 ppm EDB in fumigated oats at several weeks post-fumigation. Wit et al. (1969) fumigated wheat for 10 days with 4 kg EDB per 1000 bushels. At 12 weeks post-fumigation, they found on average 5 ppm EDB in whole grain and 2 ppm EDB in flour, 18 ppm EDB in bran, and .002 ppm in bread derived from the whole grain. McMahon (1971) fumigated wheat with an admixture of CT, CD, EDB, and methylene chloride (70.5:16.5:6.6:6.4 w/w). The admixture was applied at 3.8 liters per 1000 bushels i.e. 550 g EDB per 1000 bushels. Residues of 2.5 ppm EDB in whole grain and 1.3 ppm EDB in milo at 2 and 3 months post-fumigation, respectively, were reported.

Rains and Holder (1981) quantified residues of EDB in flour and biscuit samples that would have been used in a school lunch program. They found up to 4.2 ppm EDB and 0.3 ppm EDB in flour and biscuits, respectively. Out of 22 flour and 22 biscuit samples analyzed, 5 samples of each exceeded current tolerance limits for EDB.

In 1984, the EPA conducted a survey on residues of EDB in grain products. The data were obtained from several government and industry sources. See Table 1 for a summary. Thirty to 75.2% of raw grains, 17.4 to 69.3% of milled grain products, and 6.0 to 39.5% of ready-to-eat grain products analyzed contained detectable residues of EDB. Detection limits were 1 ppb or less. Residues detected ranged from ND to more than 10,000 ppb in raw grains, ND to 990 ppb in milled grains, and ND to 51.5 ppb in ready-to-eat grain products. The percentage of samples analyzed which exceeded current tolerance limits for EDB was not reported. In this survey, the EPA also

estimated that prior to bans against use of EDB as a pesticide: 1) 60% of wheat products marketed in the United States contained detectable EDB and 2) the public was exposed to 8.6×10^{-5}mg EDB per kg of diet per day from wheat products alone. No estimates were given as to time needed for EDB-contaminated food products to pass from the market (Brown 1984, EPA 1984).

All studies reviewed have reported a marked decrease in EDB residues after aeration, processing, or cooking. These losses could be due to: 1) evaporation of physically sorbed EDB and/or 2) conversion of physically sorbed EDB to chemically sorbed EDB. If the latter occurs, then the concentration of free bromide should increase. Olomucki and Bondi (1955) aerated EDB-fumigated sorghum for 45 days. During that time, free bromide residues increased 10-15% while EDB residues *per se* decreased 95-99%. Morris and Fuller (1963) stored an EDB-fumigated laying mash in a non-hermetically sealed container for seven days. EDB residues *per se* dropped about 40% while free bromide increased 5-10%. Ambient temperature did not fluctuate significantly during the study period in either trial. Thus, losses of EDB during aeration and storage of grain are primarily due to evaporation of physically sorbed EDB. It is not known whether losses of EDB in grain due to high temperature are a result of evaporation or chemical sorption. It is likely, however, that more physically sorbed EDB is converted to chemically sorbed EDB during exposure to high temperatures than during aeration.

In summary, the concentration of EDB *per se* in grain after

Table 1. Summary of the EPA survey on EDB residue data in grains and grain products (Brown, 1984).

Commodity	EDB residues found: No. of samples	EDB residues found: % detectable residues	Range of residues[1]: minimum	Range of residues[1]: maximum	Range of residues[1]: Average residue	Range of residues[1]: Median residue
Raw Grain Products						
Wheat	862	75.2	ND[2]	1842	40.3	4.0
Corn	290	60.7	ND	>10,000	55.8	1.4
Other	112	29.5	ND	>10,000	109.9	ND
Milled Grain Products						
Wheat	638	69.3	ND	450	14.4	2.0
Corn	303	55.1	ND	990	44.1	1.5
Other	46	17.4	ND	128	4.0	ND
RTE Grain Products[3]						
Wheat	272	21.7	ND	49.4	2.3	ND
Corn	86	39.5	ND	51.5	4.0	ND
Other	100	6.0	ND	3.8	ND	ND

[1] All residues are in ppb.

[2] ND = not detected, detection limit = 1.0 ppb.

[3] RTE = ready-to-eat.

fumigation is dependent on the grain's chemical composition and varies with dose, length of exposure, and amount of processing, cooking, and aeration. Directly fumigated grains contain the highest concentration of EDB and residues decrease upon further processing. All but one of the studies reviewed reported residues of EDB which exceeded the current tolerance limits.

IV. Residues of Ethylene Dibromide in Tissues and Blood of Rats and Chicks

In contrast to grains, little work hsa been done to quantify residues of EDB in tissues of animals acutely or chronically exposed to EDB. Morris and Fuller (1963) fed 2-week old chicks a diet containing 280 ppm EDB for 2 weeks. They found 118 ppm EDB in liver and 123 ppm EDB in kidney. They did not determine if residues decreased upon withdrawal from contaminated diet. Nachtomi and Alumot (1972) gave a single oral dose of EDBV to chicks (14 mg EDB per 100 g of body weight) and rats (22 mg EDB per 100 g of body weight). At 5 minutes post-dose, chick blood and liver contained 4 ug EDB per ml and 24 ug of EDB per 100 g of body weight, respectively, while rat blood and liver contained 7.1 ug EDB per ml and 70 ug EDB per 100 g of body weight, respectively. EDB was non-detectable (detection limit was less than 2 ug) in rat blood, rat liver, chick blood, or chick liver by 2, 13, 24, and 24 hours post-dose, respectively. This indicates an efficient metabolizing process.

V. Toxicology of Ethylene Dibromide in Poultry

The effects of EDB on growth, production, and reproduction in

poultry have been studied. Morris and Fuller (1963) fed 2-week old male chicks diet at 40 ppm EDB for 2 weeks and observed a decrease in growth rate, feed consumption, and feed efficiency. In a paired feeding trial, the decrease in growth rate was found not only to be a result of reduced feed intake, but was also due to a growth depressant effect of EDB. Alumot et al. (1968) pair fed three-day old male chicks diet at 0, 80, or 180 ppm EDB for 12 weeks and found EDB reduced feed consumption but not growth.

Bondi et al. (1955) fed diet at 10, 25, or 60 ppm bromide to hens. Bromide was incorporporated into the diet by fumigating sorghum with EDB. Then, free (physically sorbed) EDB was extracted from the sorghum, and the grain was blended into a laying mash. Thus, the diets fed to hens contained only chemically sorbed EDB and free bromide and did not contain free EDB. Diets were fed to hens for 16 weeks, and it was reported that there was no effect on egg production or egg weight. Bondi et al. (1955) also fed hens diet at 10 ppm EDB per se for 12 weeks and reported a decrease in egg weight. Thus, they showed that it is physically sorbed, chemically unaltered EDB which is responsible for toxic effects in poultry.

Fuller and Morris (1962) dosed hens orally with 0.5, 1, 2, 4, or 8 mg EDB per hen per day (mg EDB/h/d) over several weeks. Hens averaged 100 grams of intake per day so the doses were equivalent to 5, 10, 20, 40, or 80 ppm EDB in the diet. EDB was dissolved in a water-ethanol solution and injected into the crop daily as follows: 12 weeks of EDB injections followed by 8 weeks of non-contaminated injections followed by 12 weeks of EDB injections. At the end of the

treatment period, egg production, and egg weights of EDB-treated birds were compared to controls. Doses from 0.5 to 4 mg EDB/h/d (5 to 40 ppm EDB) had no effect on egg production but hens which received 8 mg EDB/h/d (80 ppm EDB) produced 25% fewer eggs than controls. All doses of EDB reduced egg weight. The loss in egg weight followed a dose-response pattern. Eggs from hens that received 0.5 mg EDB/h/d (5 ppm EDB) weighed 5% less than controls, whereas eggs from hens that received 8 mg EDB/h/d (80 ppm EDB) weighed 40% less than controls. Hens were fed non-contaminated diet for several months after EDB treatment was complete. Egg production returned to normal after 12 weeks but egg weight did not equal that of controls until 6 to 10 months post-treatment. In 1963, Fuller and Morris repeated the 1962 study with one change. Hens were fed EDB in the diet instead of via daily oral doses. As in their 1962 study, it was found that 5 ppm EDB reduced egg weights, but egg production was not affected at doses less than 80 ppm EDB. They also found that at all doses of EDB there was no effect on feed consumption, body weight, or mortality.

Olomucki (1957) showed that decreases in egg weight were due to impaired follicle growth. Fuller and Morris (1962) found that follicles in ovaries of EDB treated hens were only partially developed. Alumot and Mandel (1969) showed that the impaired growth of follicles was not due to impaired synthesis or release of gonadotropic hormones. Alumot and Harduf (1971) found that the decrease in egg size may be related to impaired follicular uptake of serum proteins (albumin and globulin). Hens were fed 100 ppm EDB until egg weight had dropped to 33% below controls. Then, follicular uptake of ^{125}I labeled serum proteins was

measured. Uptake of serum proteins per whole yolk or per unit of membrane area was only half that of controls. The authors hypothesized that membrane permeability was impaired.

Alumot et al. (1968) conducted several feeding trials which assessed the effects of EDB on reproduction in chickens. In 2 of the trials they fed male chicks diet at 0, 80, or 180 ppm EDB and female chicks diet at 40 ppm EDB from hatch until sexual maturity and found no delay in age of onset of egg or sperm production. In another trial they fed adult males 150 or 300 ppm EDB for 12 months and found no effects on spermiogenic activity, spermatozoa count, or testes weight but a decrease in comb weight was reported. Semen from those males was used to artificially inseminate control females and no effect was observed on fertility or hatchability of eggs. In a final trial laying hens were fed diet at 100 ppm EDB for 4 weeks after which they were artificially inseminated with semen from control males. Only 12% of eggs laid by EDB-treated females were fertile. None of the fertile eggs hatched.

Westlake (1981) orally dosed Japanese quail (Coturnix coturnix) in order to determine the LD_{50} and LC_{50}. The single oral LD_{50} for EDB in Japanese quail was 130 mg EDB per kg of body weight. A 95% confidence interval on the LD_{50} ranges from 107.4 to 157.3 mg EDB per kg of body weight. For a five day exposure, the chornic LC_{50} for EDB in Japanese quail was 11.1 mg EDB per bird per day. Quail consumed 6.73 grams of diet per day. Therefore the LC_{50} is equivalent to diet at 1650 ppm EDB. A 95% confidence interval on the LC_{50} ranges from 8.9 to 13.9 mg EDB per bird per day (1320 to 2020 ppm EDB in diet).

In summary, the only no-effect levels determined for EDB in poultry have been for egg production and male reproduction. These were found to be, respectively, 40 ppm and 150 ppm EDB or less in the diet. The dietary levels of EDB which have no effect on egg weight, growth, and female reproduction in poultry have not been determined but are less than 5 ppm, 40 ppm, and 100 ppm, respectively.

Current tolerance limits for EDB only allow grain containing less than these levels to be marketed. Therefore, it is not known if prolonged consumption of grains containing less EDB than currently allowed by tolerance limits would have any significant effect on egg weight, growth, fertility, or hatchability in poultry.

VI. Toxicology of Ethylene Dibromide in Mammals

A. LD_{50} and LC_{50}

The LC_{50} of EDB in mammalian species has not been determined. The acute oral single LD_{50} for guinea pigs, male rats, female rats, and female mice is 110, 146, 117, and 420 mg EDB per kg of body weight (Rowe et al. 1952).

B. Metabolism of EDB in rats

The metabolic half-life of EDB in intravenously injected rats is 2 hours. EDB is metabolized in rats by: 1) conjugation with glutathione (GSH) and/or 2) oxidative dehalogenation (Figure 1). GSH conjugation occurs more frequently than oxidative dehalogenation, is catalyzed by GSH S-transferases, and occurs primarily in the liver. One or 2 GSH's can be transferred to EDB. If 2 are transferred, the

resultant compound, S-S'ethylene-bis(glutathione) is split hydrolytically to form S-(β-hydroxyethyl) glutathione (HEG) or the sulphoxide of HEG. If only one GSH is transferred to EDB, then HEG and its sulphoxide are formed directly. Both routes to HEG result in the release of 2 bromides which are excreted in urine. HEG and its sulphoxide can either bind to polynucleotides or be further metabolized in the kidney by a 2-step process. First, glutamic acid and glycine are removed from the GSH portion of HEG to form S-(β-hydroxyethyl)cysteine (HEC) or the sulphoxide of HEC. Then, HEC and its sulphoxide are metabolized to S-(β-hydroxyethyl) mercapturic acid (HEM) and its sulphoxide. HEM is the primary metabolite of EDB. HEC, HEM and their sulphoxides are excreted in urine and bile (EPA 1977, Nachtomi et al. 1966, Nachtomi 1970, Shih and Hill 1981).

Oxidative dehalogenation of EDB occurs primarily in the liver. The reaction is catalyzed by the microsomal oxidase that is induced by phenobarbital. The end-product is 2-bromoacetaldehyde that either: 1) binds to proteins or 2) converts to 2-bromoacetic acid. 2-bromoacetic acid is excreted in urine (Shih and Hill 1981).

C. Mutagenicity and oncogenicity of EDB in rats and mice

Several researchers (Rannug 1980, Anonymous 1977, Dept. of Labor 1983) have reviewed in detail the mutagenic and oncogenic actions of EDB. EDB acts as a mutagen by covalently binding to DNA via an alkylation reaction which releases a bromide molecule. The result is formation of a "half-mustard" reagent that can undergo a

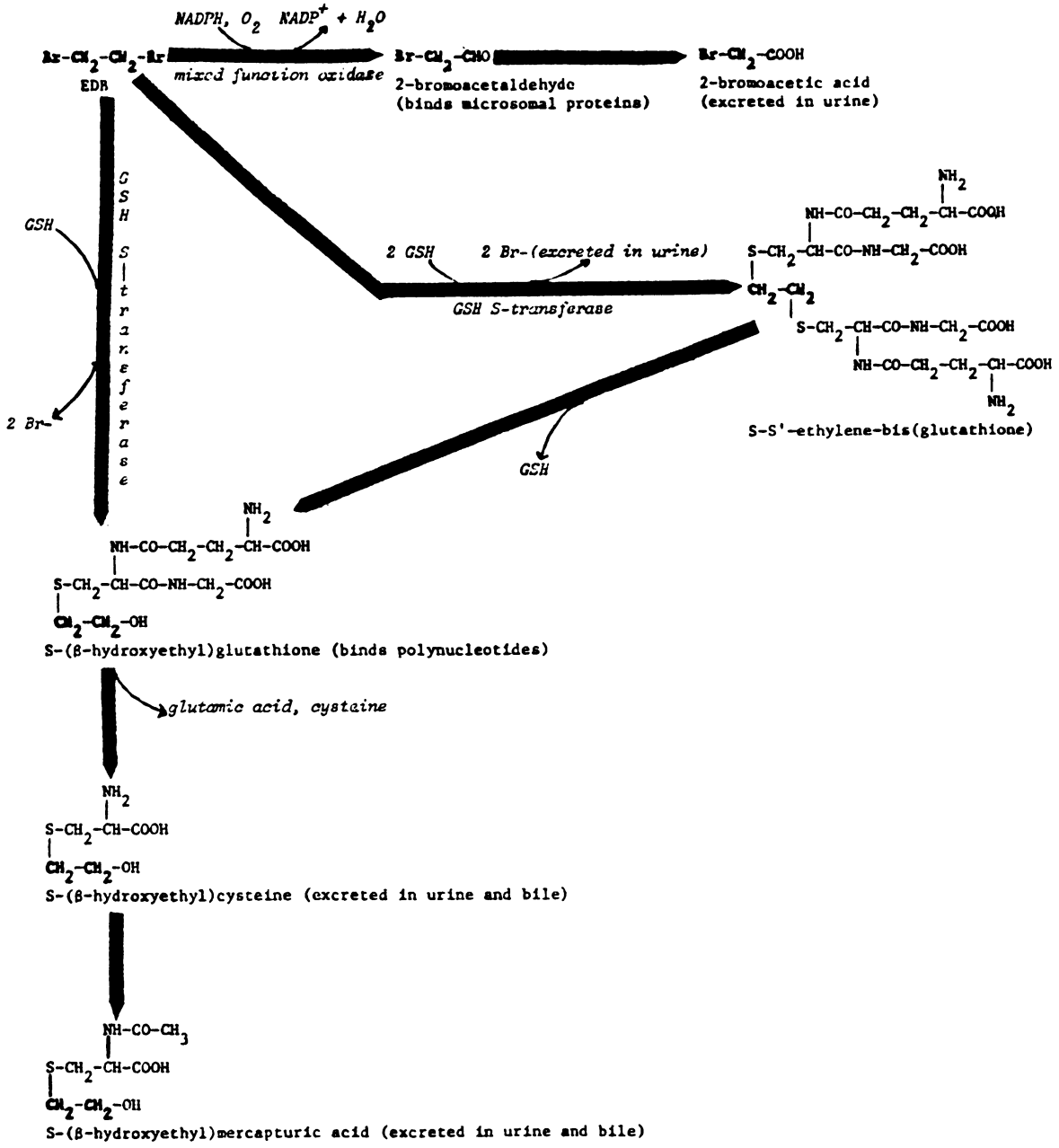

Figure 1. Metabolism of EDB in rats.

second alkylation. Binding of EDB to DNA in this manner causes: 1) separation of strands, 2) base pair transitions, and 3) single strand breaks. EDB can be activated to a mutagen of greater potency by conjugation to GSH. It is thought that EDB might be responsible for contact tumors while its GSH conjugate might be responsible for tumors in remote organs.

EDB has also been found to be a potent carcinogen. Tumors have been observed in the forestomach, adrenals, mammary glands, lungs, and nasal cavities of rats and mice exposed to EDB by inhalation, intraperitoneal injections, gavage, or dermal routes. Nitsche et al. (1981) exposed male and female rats to 0, 3,10, and 40 ppm airborne EDB as follows: 6 hours per day and 5 days per week for 13 weeks. They found that the no-effect level for tumor incidence after inhalation of EDB as outlined is 3 ppm airborne EDB. The no-effect levels for tumor incidence after dermal or oral exposure have not been determined.

D. Effects of EDB on reproduction

The EPA published a detailed review on the reproductive effects of EDB in rats, bulls, cows, sheep, and mice (EPA 1977). EDB has been found to interfere with both male and female reproductive processes. For example, Amir and Lavon (1976) dosed bulls with 4 mg EDB per kg of body weight on alternate days over a 20-day period and found that sperm production and motility were reduced while the number of sperm that had misshapen heads was increased. Short et al. (1976) exposed pregnant rats and mice to 32 ppm airborne EDB for 23 hours per day during days 6 to 15 of

gestation. It was reported that EDB decreased fetal weight, the number of implants per dam, and the number of fetuses per dam. The no-effect levels for male and female reproduction in mammals via exposure by oral, inhalation, or dermal routes have not been determined.

E. Toxicology of EDB in humans

Humans are exposed to EDB by ingestion, inhalation, or dermal contact. Ingestion of EDB is a result of consumption of EDB-contaminated grain or fruit while inhalation exposure to EDB comes from leaded gasoline fumes which can contain trace quantities of EDB. Both inhalation and dermal exposure occur occupationally during production and/or application of EDB. The Occupational Safety and Health Administration has published a detailed review on occupational exposure to EDB (Dept. of Labor 1983). Several other studies have reviewed the toxicology of EDB in humans. Olmstead (1960) reported that a woman who had ingested a single dose (4.5 ml) of EDB experienced vomiting, abdominal pain, diarrhea, and nausea. She died 54 hours after ingestion. An autopsy showed massive centrilobular liver necrosis and damage to tubular epithelium of kidneys. Dermal contact with EDB has been found to cause severe burns (Peoples et al. 1978). Studies that have surveyed populations that are exposed to EDB have only given limited evidence that EDB decreases fertility or increases the risk of cancer (Ott et al. 1980, Takahashi 1981, Wong 1979). However, in 1983, using a one-hit carcinogen model derived from animal carcinogen studies with EDB, the EPA predicted that the

levels of EDB in the nation's food supply at that time would lead to an additional 3 cancer deaths per 1000 people. They also predicted that lifetime occupational exposure to 0.4 ppm airborne EDB would lead to an almost 100% chance of developing cancer. It is these estimates which caused the EPA to ban uses of EDB as a pesticide. Much controversy surrounds the EPA's estimates (Ramsey et al. 1979), and it is unclear at this time what amount or length of exposure to EDB in the diet or through inhalation will increase cancer risk to the general public.

VII. Summary

It has been found that EDB residues per se are sorbed by grain during fumigation. This results in EDB exposure to the general public through consumption of EDB-contaminated grain. Therefore, much work was done to categorize the mutagenic, oncogenic, and reproductive effects of EDB in animals. The results of this research were used to estimate possible toxic effects of EDB in humans. However: 1) most of the research was conducted with levels of EDB that are much higher than are usually found in food products after fumigation with EDB, 2) few no-effect levels have been determined, and 3) little work has been done to quantify residues of EDB in tissues of food-producing animals after consumption of EDB-contaminated grain. If the latter occurs, it would represent another route for EDB exposure to the general public. Thus, the purpose of this research was to feed chickens EDB-contaminated grain obtained from the Michigan food supply to determine if and to what extent EDB per se is deposited into tissues and eggs.

MATERIALS AND METHODS

I. Experimental Methods

A. Composition and blending of experimental diets

An EDB-contaminated diet and a non-contaminated diet were prepared. Both diets contained equal amounts of all ingredients except for flour. The EDB source was a flour confiscated by the Foods Division of the Michigan State Department of Agriculture. It and a non-contaminated cookie flour obtained from Michigan State University Food Stores were incorporated into their respective diets to account for 55.7% of the diet. The contaminated flour was confiscated on February 2, 1984 from the Amendt Milling Company of Monroe, MI and was an unbleached cake flour marketed as Honey Queen. The confiscated cake flour contained 31.1 ppm EDB. The resulting EDB diet assayed at 6.7 ppm EDB although it theoretically should have contained 17.3 ppm EDB. The non-contaminated cookie flour was a non-brominated pastry flour produced by Michigan Bakery Supply and was marketed as Cookie Maker. Both poultry diets were blended in Mix-Mill$^{T.M.}$ 250 CT Nutri Blenders. To prevent contamination of the control diet, it was blended in one Mix-Mill blender, and the EDB diet was blended in another.

B. Husbandry

Single Comb White Leghorn female chickens in their first

Table 2. Composition of experimental diets.

Ingredient	Non-contaminated flour diet[1] parts/1000	EDB-contaminated flour diet[2] parts/1000
Alfalfa, dehy, 17%	40	40
Soybean meal, 44%	240	240
Cookie flour[3]	557	0
Cake flour[4]	0	557
Limestone	72	72
Dicalcium phosphate	22	22
Corn oil	55	55
Ethoxyquin	0.125	0.125
dl-methionine	1	1
Magnesium oxide	5	5
Choline chloride, 50%	1	1
Vitamin mix[5]	3	3
Mineral mix[6]	0.5	0.5
Selenium mix[7]	0.5	0.5
Iodized salt	3	3

[1] Metabolizable energy = 2.65 kcal/g; Crude protein = 17%

[2] Metabolizable energy = 2.65 kcal/g; Crude protein = 18%

[3] Non-contaminated flour purchased from Michigan State University Food Stores.

[4] contained 31.7 ppm EDB; confiscated from the Amendt Milling Co. of Monroe, MI.

[5] Supplied per kg of diet: Vitamin A, 11,000 I.U.; Vitamin D_3, 1,100 I.C.U.; Vitamin E, 11 I.U.; Vitamin K, 22 mg; Thiamin, 2.2 mg; Riboflavin, 4 mg; Pantothenic acid, 14.1 mg; Nicotinic acid, 31.5 mg; Pynidoxine, 4 mg; Biotin, 0.1 mg; Folic acid, 1.3 mg; Choline, 13.2 mg; Vitamin B_{12}, 0.01 mg.

[6] From Calcium Carbonate Company; Supplied per kg of diet: Manganese, 60 mg; Zinc, 40 mg; Iron, 30 mg; Copper, 5 mg; Iodine, 0.5 mg.

[7] From Calcium Carbonate Co.; Supplied 0.1 mg Selenium/kg diet.

year of production were used in this study. They were obtained from a laying flock maintained at the Michigan State University Poultry Research and Teaching Center (PRTC). Control hens were housed in Anthony Hall, Michigan State University and were confined singly in 41.9x20.3x40.6 cm (LxWxH) cages with 5.1x2.5 cm wire mesh. EDB-treated hens were housed at the PRTC. They were isolated to prevent the possible spread of EDB. EDB-treated hens were confined singly in 45x45x45 cm (LxWxH) cages with 5.1x2.5 cm wire mesh. Artificial lighting was supplied to both rooms on a schedule of 16 hours light:8 hours dark, daily. The control room was maintained at 23±10°C while the EDB room was maintained at 18.3±2.8°C.

Control and EDB-treated hens received feed and water ad libitum throughout the study. Feed intake and body weights were obtained weekly. Eggs were collected daily, marked in pencil with date, cage number, and treatment, and stored in plastic egg flats at room temperature until processed for EDB residue analysis.

C. Schedule

The study consisted of a pre-experimental and an experimental time period. The experimental time period was subdivided into residue build-up and residue withdrawal time periods based on dietary treatment and expected concentration of EDB residues in eggs and tissues. See Table 3 for a summary of the schedule.

The pre-experimental period, from March 7 to March 20 (days -14 to -1 of the study), was to allow the hens to adapt to their

respective environments and to convert the hens from the usual commercial-type mash to the semi-purified type diet containing the flour. The transition of one diet to the other occurred over a 7 day period the first 2 days of which hens were fed a blend of commercial laying mash (CLM) and non-contaminated flour diet (NCFD) in a ratio of 3:1 (CLM:NCFD). This was followed by 2 days of feeding the blend at 1:1 and one day of feeding at 1:3. The next day hens were fed 100% NCFD completing the transition to the semi-purified type diet.

The feeding of the EDB-contaminated diet occurred from March 21, 1984 to April 11, 1984 (Days 0 to 21 of residue build-up). The duration of 21 days was considered sufficient to allow maximum build up of EDB in eggs and hens. On March 21, the 8 best laying hens in Anthony and the 16 best laying hens at PRTC were chosen to receive experimental diets. Hens not chosen were returned to the PRTC laying flock. The 8 hens in Anthony received the diet with non-contaminated flour, and the 16 hens at PRTC received EDB-contaminated diet from March 21 until the evening of April 10 when feed was removed. Hens were fasted for 18 hours prior to necropsy to allow feed residues to pass through the gastrointestinal tract. This eliminated the possibility that during assay procedures feed residues of EDB would contribute to the EDB residues in the chickens.

On April 11, 4 control and 8 EDB hens were randomly selected and euthanized bloodlessly with excess CO_2. Of the hens

Table 3. Experimental schedule.

	Dates	Experimental days	Number of hens	Treatment
		Pre-experimental		
Control hens	3/ 7/84-3/20/84	-14 to -1	8	None[1]
EDB hens	3/ 7/84-3/20/84	-14 to -1	16	None[1]
		Residue build-up		
Control hens	3/21/84-4/11/84	0 to 21	8[2]	Non-contaminated flour diet
EDB hens	3/21/84-4/11/84	0 to 21	16[3]	EDB-contaminated flour diet
		Residue withdrawal		
Control hens	4/11/84-5/ 2/84	21 to 42[4]	4[2]	Non-contaminated flour diet
EDB hens	4/11/84-5/ 2/84	21 to 42[4]	8[3]	Non-contaminated flour diet

[1] Hens were acclimated to the semi-purified flour diet during this period.

[2] 4 control hens were sacrificed on the final day of each specified time period, 2 of which were used for analysis of EDB residues in whole body and 2 of which were used for tissue analysis.

[3] 8 EDB hens were sacrificed on the final day of each specified time period, 4 of which were used for analysis of EDB residues in whole body and 4 of which were used for tissue analysis.

[4] Days 0 to 21 of withdrawal.

euthanized, 2 control and 4 EDB hens were randomly selected for determination of whole body EDB residues. They were vacuumed to remove dust from their feathers and then sealed in plastic bags and frozen at -20°C. From the other 2 control and 4 EDB hens euthanized, liver, kidney, abdominal fat, breast skin, and right breast muscle (pectoralis superficial) were removed for analysis of EDB residues in those tissues. The livers were weighed and had 10 grams excised for measurement of mixed function oxidase (MFO) activity. The tissues were sealed in plastic bags and frozen at -20°C. After necropsy, the EDB room was cleaned and vacuumed, thereby completing the time period for residue build-up.

Residue withdrawal extended from April 11, 1984 to May 2, 1984 (Days 0 to 21 of withdrawal). It was predicted that concentration of EDB in eggs and tissues would be reduced during this time. The remaining 4 hens in Anthony and 8 hens at PRTC were fed non-contaminated flour diet from April 11 until the evening of May 1 when feed was removed. Hens were fasted for 18 hours, and on May 2 the study was terminated when the remaining hens (4 control and 8 EDB) were euthanized bloodlessly with excess CO_2. The necropsy procedure used on April 11 was also followed on May 2, i.e., samples were obtained for whole body, tissue, and MFO analysis. The number of hens euthanized and tissue samples taken at each time period is presented in Table 3.

D. Safety methods and contaminated waste disposal

Because EDB is a xenobiotic, its use is regulated. Thus, EDB-treated hens were isolated in room 4E PRTC, and any part of

the research which involved possible contamination from EDB was conducted within room 4E. All personnel who entered 4E wore protective clothing (disposable coveralls, hair nets, masks, gloves, and plastic boots). Research equipment used in the room was rinsed with hexane to remove EDB residues. Droppings were collected on disposable plastic sheets, and all inorganic and organic waste was sealed in barrels and disposed of in accordance with state and federal laws by Michigan State University's Laboratory Animal Care Service.

II. Mixed Function Oxidase Assay

A. Introduction

There are two classes of xenobiotics (XB) which induce activity in mixed function oxidase (MFO) enzymes: phenobarbital-type inducers and 3-methylcholanthrene-type inducers. Activity of aminopyrine N-demethylase (AND) is measured to determine if a XB is a phenobarbital-type inducer, and activity of aryl hydrocarbon hydroxylase (AHH) is measured to determine if a xenobiotic is a 3-methylcholanthrene-type inducer. Both enzymes are found in the cytosol of liver cells associated with rough endoplasmic reticulum (Bresnick 1978). AND and AHH activity were accounted for in microsomes (rough endoplasmic reticulum) and were related to protein content of the microsomes. The methods for determining AND and AHH activity were adapted from Anders and Mannering (1966) and Van Cantfort et al. (1977), respectively

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MFO

B. Miscellaneous

See Appendix E for preparation of reagents. Reagents were prepared the day before the livers were procured and the microsomes were isolated. During the assay, Gilson Pipetman adjustable-volume pipets (Models K-79-16143, K-79-11742, K-79-11158, and K-79-16907) and Rainin disposable pipet tips (types RC20, RC200 and RC2000) were used to pipet volumes between 20 ul and 5 ml. Volumes of 5 ml or greater were dispensed with graduated cylinders or a Lab Industries Repipet®. Samples were on ice during all steps of the assay except incubation. See Appendix F for raw data obtained during crude protein determination and assay termination.

C. Isoation of microsomes

Livers were excised from hens, weighed, and profused with cold 150 mM KCl. Approximately 10 g of each profused liver was placed into a polycarbonate centrifuge tube and minced into small pieces with a pair of scissors. Approximately 20 ml (2 times the wet weight of the liver sample) of homogenizing buffer was then added to the centrifuge tube, and the liver was homogenized for 5 seconds, twice at speed 5 with a polytron homogenizer (Type PT 10 OD). Inbetween samples, connective tissue was cleaned out of the polytron blade, and the blade was rinsed with double-distilled water (DD H_2O). After homogenization, the samples were spun for 20 minutes at 12,000 rpm in a Sorvall® Superspeed RC-2 Centrifuge with SA-600 rotor. The resultant supernatant was poured through a

triple layer of cheesecloth into a thick-walled polycarbonate centrifuge tube and spun for 75 minutes at 30,000 rpm in a Beckman L2-65B ultracentrifuge with type 30 rotor. The supernatant was discarded and the microsome pellet was left in the centrifuge tube. Ten milliliters of 200 mM Tris-HCl was added to the centrifuge tubes, the tubes were covered, and the samples were stored overnight at 4°C. The following day, the pellet was scraped off the side of the centrifuge tube with a glass rod and suspended into 200 mM Tris-HCl by homogenization with the polytron at speed 5 for 2 seconds.

D. Determination of crude protein in microsomes

After the microsome pellets were suspended into 200 mM Tris-HCl, the concentration of crude protein in the microsomes was determined by the Biuret method (Gornall et al. 1949). Solutions of bovine serum albumin (BSA) at 0, 3, and 5 mg were used to establish a dose-response curve of mg of protein versus spectrophotometric absorbance from which the weight (mg) of protein in microsome samples was calculated. The steps involved were:

1. Duplicates of each standard curve solution and each microsome sample were analyzed.
2. Volumes of DD H_2O, 6% NaOH, and 180 mM KCl were added to standard curve and microsome sample test tubes as outlined in Table 4.
3. BSA was added to standard curve test tubes as outlined in Table 4.
4. 100 ul of microsomes was added to each microsome sample test tube.

Table 4. Volumes of reagents used to establish a three point standard curve for the biuret protein determination assay.

Reagents[1]	Protein - mg			
	Unknowns[2]	0[3]	3[3]	5[3]
Microsomes - ml	0.1	0.0	0.0	0.0
Bovine serum albumin - ml	0.0	0.0	0.6	1.0
Biuret - ml	0.2	0.2	0.2	0.2
Double-distilled H_2O - ml	1.9	1.9	1.4	1.0
6% NaOH - ml	2.0	2.0	2.0	2.0
150 mM kcl - ml	0.0	0.1	0.0	0.0
Total volume - ml	4.2	4.2	4.2	4.2

[1] See Appendix E for preparation of reagents.

[2] Refers to microsome samples for which protein concentration was being determined.

[3] Standard dilutions used to establish a dose-response curve of mg of protein versus spectrophotometric absorbance from which concentration of protein in microsome samples was calculated.

5. 200 ul of Biuret was added to each test tube.

6. All test tubes were vortexed for 15 seconds at full speed with a K-550-G Vortex-Genie.

7. Color was allowed to develop for 10 minutes.

8. Absorbance of each sample was read in a Gilford Stasar II 1367x5 spectrophotometer at λ = 540 nm. DD H_2O was used as zero.

9. From the standard curve solutions a dose-response line was calculated for x = mg protein and y = absorbance.

10. The amount of protein (mg) in 100 ul of microsomes was inversely predicted from the dose-response line. After duplicates were averaged, this number was divided by 0.1 to obtain mg protein/ml microsomes.

11. Each microsome sample was then diluted with 200 mM Tris-HCl so that there was 1 mg protein/200 ul microsomes.

E. Incubation of microsomes with substrate

After microsome samples were diluted to 1 mg protein/200 ul, microsomes were incubated with aminopyrine and benzo(α)-pyrene substrates so that activity of AND and AHH, respectively, could be measured.

1. The water bath in a Dubnoff Metabolic Shaking Incubator was preheated to 37°C.

2. For the AND assay, 2 samples and 2 blank 12x75 mm test tubes were labeled for each microsome sample. Two 12x75 mm test tubes were labeled for each point of the AND standard curve (Table 5).

3. For the AHH assay, 2 samples and 2 blank 8 ml scintillation vials were labeled for each microsome sample.

4. 16 ml of 200 mM Tris-HCl, 1.28 ml of glucose-6-phosphate (G6P), 640 ml of 200 mM $MgCl_2$ and 64 ul of glucose-6-phosphate dehydrogenase (G6PD) were blended to make a premix. In the premixes the reagents must be in the ratio 250:20:10:1 (Tris-HCl:G6P:$MgCl_2$:G6PD).

Table 5. Volumes of reagents used to establish a standard curve for the aminopyrine N-demethylase assay.

Reagents[1]	Standard curve solutions - mM formaldehyde			
	0[2]	3[2]	6[2]	12[2]
30 mM formaldehyde - µl	0	100	200	400
Double-distilled water - µl	1000	900	800	600

[1] See Appendix E for preparation of reagents.

[2] 30 mM formaldehyde (CH_2O) and double-distilled water were blended as outlined in the table. Then 20 µl of each solution was used to develop a standard curve of X = nmoles formaldehyde and Y = spectrophotometric absorbance. 20 µl of 3 mM CH_2O = 60 nmoles CH_2O; 20 µl of 6 mM CH_2O = 120 nmoles CH_2O; 20 µl of 12 mM CH_2O = 240 nmoles CH_2O.

5. 280 ul of premix was pipetted into AND and AHH blank tubes and vials and AND standard curve tubes.

6. 16 mg of β-nicotinamide adenine dinucleotide phosphate (NADP, Sigma, N-0505) was blended into the remaining premix, i.e. 0.5 mg of NADP was added for each 280 ul of premix remaining.

7. 280 ul of NADP premix was added to AND and AHH sample tubes and vials.

8. A four point AND standard curve was established as outlined in Table 5 by pipetting 20 ul of each standard solution into its corresponding test tube. Then, 200 ul of 150 mM KCl was added to each standard curve test tube.

9. 20 ul of tritium-labeled benzo(α)pyrene was pipetted into three 8 ml scintillation vials labeled as total count vials. 5 ml of benzo(α)pyrene cocktail was added, and the vials were capped and saved until assay termination.

10. 200 ul of microsomes was pipetted into AND blank and sample tubes and AHH sample vials. Tubes and vials were placed in the water bath.

11. Sample, blank, and standard curve tubes and vials were oscillated in the water bath for 5 minutes at 37°C and 60 rpm.

12. Oscillation was stopped, a timer was set for 30 minutes, and at 10 second intervals, 20 ul of aminopyrine substrate was pipetted into AND blank and sample tubes. Substrate was never added to AND standard curve tubes. Also at 10 second intervals, 20 ul of benzo(α)pyrene substrate (BP) was added to AHH blank and sample tubes. After substrates were added, tubes and vials were incubated at 37°C and 60 rpm for the time that remained out of the 30 minutes.

F. Assay terminations and calculation of enzyme activity

After the microsomes were incubated with substrate, AND and AHH enzymatic reactions were terminated and amount of product formed was measured as follows:

I. AND termination and activity calculations

1. After 30 minutes of incubation, at 10 second intervals, 1 ml of 20% $ZnSO_4$ was added to AND blank, sample, and standard curve tubes.

2. Tubes were removed from the water bath, and 1 ml of saturated BaOH was pipetted into each tube.

3. Tubes were vortexed at full speed for 15 seconds.

4. Tubes were centrifuged at full speed for 30 minutes in a Sorvall® GLC-4 centrifuge.

5. The water bath was heated to 60°C.

6. After tubes had finished spinning, 7 ml of supernatant and 1 ml of nash reagent were pipetted into a 12x75 mm test tube, and tubes were vortexed at full speed for 15 seconds.

7. Samples were heated in the water bath for ten minutes and then cooled to room temperature.

8. Absorbance was read in the Gilford spectrophotometer at λ = 412 nm. DD H_2O was used as zero.

9. From the absorbance of the standard curve solutions, a dose-response line was calculated for x = nmoles formaldehyde produced/30 minutes and y = absorbance.

10. The amount of formaldehyde produced by AND in 30 minutes in nmoles was inversely predicted from the dose-response line. Duplicates were averaged, and blank values were subtracted from sample values. The resultant number was divided by 30 to obtain net nmoles formaldehyde produced by AND/mg microsomal protein/minute of incubation.

II. AHH termination and activity calculations

1. After 30 minutes of incubation, at10 second intervals, 1 DMSO-KOH was added to AHH blank and sample vials.

2. Vials were removed from the water bath and 200 ul of microsomes were added to AHH blank vials.

3. 5 ml of glass-distilled hexane was added to blank and sample vials.

4. Vials were rotoracked for 20 minutes at full speed in a Fisher 343 Roto-Rack.

5. The hexane layer was aspirated. Any emulsion formed during rotoracking was not aspirated.

6. Steps 3 and 4 were repeated.

7. The hexane and emulsion layers were aspirated.

8. 500 ul of the DMSO-KOH phase was transferred to an 8 ml scintillation vial, 5 ml of benzo(α)pyrene cocktail was added, and vials were capped.

9. AHH blank, sample, and total count vials were counted for 10 minutes on channel 11 in a Searle 6870C Isocap/300 Temperature Controlled Liquid Scintillation Counter.

10. After the vials were counted, 100 ul of tritium-labeled toluene (New England Nuclear, NES-006, 2.07×10^5 dpm/100 ul in April 1984) was added to each vial, and vials were recounted for 10 minutes on channel 11. B channel counts per minute (cpm) before and after toluene spike were calculated by dividing by 10. Efficiency was calculated for sample, blank, and total count vials as follows:

$$\text{Efficiency} = \frac{\text{cpm after toluene spike} - \text{cpm before toluene spike}}{2.07 \times 10^5 \text{ dpm}}$$

11. AHH produces hydroxylated benzo(α)pyrene (BP-OH) as an end product. The pmoles of BP-OH produced by AHH/mg microsomal protein/minute of incubation in sample and blank solutions was calculated as follows:

$$\text{pmoles BP-OH produced by AHH/mg protein/minute} =$$

$$\frac{\text{sample or blank cpm} \times \frac{1}{\text{efficiency}}}{\text{average cpm in 20 ul BP} \times \frac{1}{\text{efficiency}}} \times \frac{64{,}000 \text{ pmoles BP/20 ul}}{1}$$

$$\times \frac{3}{1} \times \frac{1}{1 \text{ mg protein in 200 ul microsomes}} \times \frac{1}{30 \text{ minutes}}$$

12. Duplicates were averaged, and net pmoles BP-OH produced by AHH/mg microsomal protein/minute of incubation was calculated by subtracting blanks from samples.

III. Headspace GC Analysis of EDB Residues in Egg, Tissues, and Diet

A. Introduction

Headspace GC was developed as a means of accurately

quantifying trace volatiles in solid matrices. One application of headspace analysis involves dissolving the solid in a liquid in a gas-tight vial. The resultant solution is heated driving volatile compounds into the headspace of the vial. After thermodynamic equilibrium between the gaseous and liquid phase is reached, an aliquot of the gaseous phase is swept onto a column for analysis (Vitenberg et al. 1974). Because EDB is volatile under the conditions described, headspace analysis was chosen as the method for quantification of EDB residues in egg, tissues, and diet.

B. Headspace gas chromatograph and integrator conditions

Residues of EDB in egg, tissues, and diet were determined with a Perkin Elmer F45 headspace GC (HSGC) equipped with: 1) a ^{63}Ni electron capture detector emitting a 3.0 mV signal, 2) a 6'x1/8" i.d. glass column containing 1% SP-1000 liquid phase on 60/80 mesh Carbopack B solid support, and 3) a Hewlett Packard 3390A integrator. Analysis temperatures were: 1) needle = 150°C, 2) injector = 150°C, 3) column = 160°C, 4) detector = 200°C, and 5) automatic turntable oil bath = 90°C. Argon:methane (9:1) was the purge gas for egg, tissue, and diet samples and was the carrier gas for egg samples while helium (99.99% pure) was the carrier gas for tissue and diet samples. Gas flow rate was 30 cc/minute for both carrier and purge. See Table 6 for integrator parameters.

C. General procedure

Egg, tissue, or diet samples were weighed with a Mettler top-loading balance into a 24 ml Perkin Elmer crimptop vial. A

Table 6. Hewlett Packard 3390A integrator conditions.

Parameter	Egg	Whole body	Skin	Fat	Kidney	Liver	Muscle	Flour	Diet
Zero - mm	5	5	5	5	5	5	5	5	5
Attenuation	-1	1	2	2	1	0	0	6	6
Chart speed - cm/min	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Peak width	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
Threshold	-1	1	1	1	0	-1	0	1	1
Area reject	100	100	100	100	100	100	100	100	100
Peak type	BB[1]	TS[2]	TS	TS	TS	TS	TS	BB	BB

[1] Baseline to baseline.
[2] Tangent skimmed.

Tipet automatic transfer pipet was used to dispense 20 N H_2SO_4 into the vial. EDB standard (for spiked samples only) was then pipetted into the vial with a Hamilton 701N microliter syringe. Vials were sealed with teflon-liquid washer septa and metal seals. Samples were digested at 90°C in the automatic turntable of the HSGC. After digestion, the vials were shaken manually, replaced in the turntable, and heated at 90°C until EDB was in equilibrium between the gas and liquid phases. Then, an aliquot of gas was injected into the column by the HSGC automatic injector. This method required that for egg, tissues, and diet several analysis parameters had to be determined: 1) identification of the EDB peak on chromatograms, 2) weight of sample and volume of acid, 3) digestion time, 4) equilibration time, and 5) analysis time.

The EDB peak on egg chromatograms was identified by obtaining chromatograms of the following samples: 1) air, 2) 5 ml 20N H_2SO_4, 3) 5 ml 20 N H_2SO_4 + 5 ul methanol, 4) 5 ml 20 N H_2SO_4 + 5 ul methanol + 5 ng EDB, 5) 5 ml 20 N H_2SO_4 + 5 ul methanol + 2 g control egg, and 6) 5 ml 20 N H_2SO_4 + 5 ul methanol + 2 g control egg + 70 ng EDB (Figure 2). The peak at retention time 5.20 or 5.21 in the chromatograms of Figure 2 is found only on chromatograms of samples which contained EDB. Therefore, it was identified as the EDB peak. This procedure was also followed when identifying the EDB peak on chromatograms of tissues and diets.

Determining the weight of sample and volume of acid to use for egg, tissues, and diet involved determining what volume of

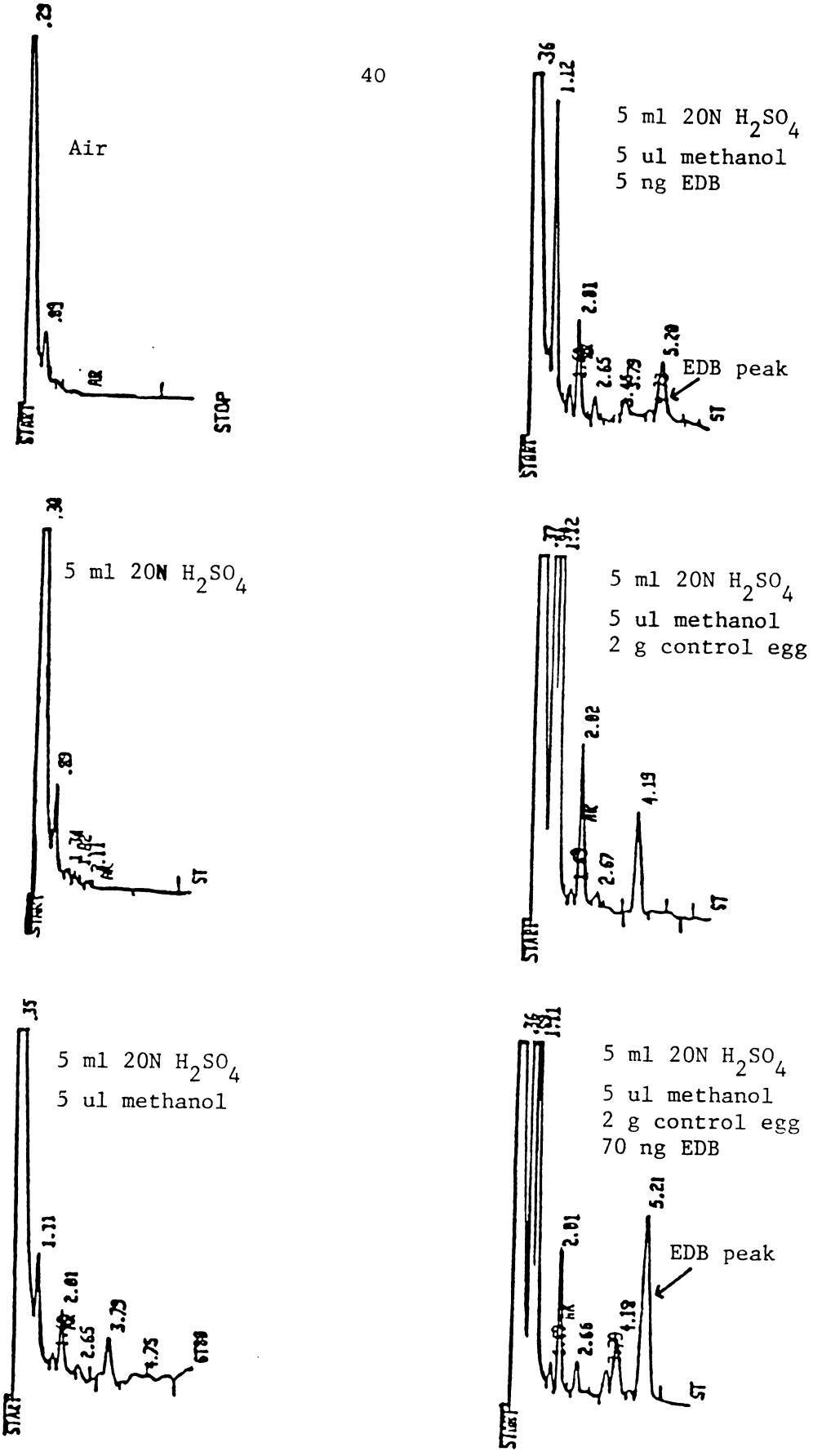

Figure 2. Identification of EDB peak on chromatograms using egg as an example.

headspace in the sample vial would give optimum resolution of the EDB peak. Sample weight had to be great enough to detect EDB while acid volume had to be great enough to digest the sample and to keep the sample in solution, but enough headspace was needed for optimum partition of EDB into the gaseous phase. Several combinations of weights and volumes were tried until the weight of sample and volume of acid which gave optimum resolution and detectability had been determined.

Sample digestion was considered complete when it appeared that all particles had been broken up and were in solution.

Equilibration time, the time needed for maximum partition of EDB into the headspace, was determined for egg, tissues, and diet as follows. Twelve control samples were spiked with equivalent amounts of EDB, digested, heated at 90°C (equilibrated), and injected onto the column at 15 minute heating intervals up to 180 minutes. Integrated peak area for EDB was recorded for each time. The point in time at which peak area plateaued was chosen as the minimum time needed for equilibration.

Analysis time, the time needed for a sample to completely pass through the column, was determined in egg, tissues, and diet as follows. A control sample was spiked with EDB, digested, equilibrated, and injected. The point in time at which peaks no longer appeared on the chromatogram was chosen as minimum analysis time.

D. Preparation and storage of EDB standard solutions

Calibration curves in egg, tissues, and diet were developed by spiking samples with EDB (Aldrich Gold Label; 99% pure; 24,065-6) dissolved in reagent grade methanol (CH_3OH). A stock solution prepared at 15 mg EDB per 50 ml CH_3OH was diluted with CH_3OH to develop standard solutions used to spike samples. Stock and standard solutions were prepared fresh weekly, stored in 50 ml volumetrics at -25°C, and warmed to room temperature before use.

E. Analysis of EDB residues in egg

Eggs collected from hens fed experimental diets were weighed individually with a Mettler top-loading electronic balance. Then egg contents (yolk + albumen) were pooled by day (over hens) within treatment for experimental days 1 to 14 and 22 to 42 (1 to 21 of withdrawal) and were pooled by hen within treatment over experimental days 15 to 21. This pooling regimen was followed because it was expected that concentration of EDB in eggs would increase from days 1 to 14 of feeding EDB-contaminated diet, be at maximum concentration from days 15 to 21 of feeding EDB-contaminated diet, and would decrease during days 1 to 21 of withdrawal from EDB-contaminated diet. See Tables 7 and 8 for a summary of how eggs were pooled. Samples were pooled (after egg contents had been broken out of the shell) by homogenization in a Waring blender at low speed for 15 seconds. Homogenized samples were stored at -20°C in glass bottles with screw caps. Prior to

Table 7. Summary of pooling method for eggs collected days 1 to 14 and 22 to 42[1].

Day	Diet	# of hens that laid[3]	
		Control treatment	EDB treatment
1	EDB-contaminated[2]	7	15
2	"	6	4
3	"	7	14
4	"	7	10
5	"	5	9
6	"	4	7
7	"	8	13
8	"	7	9
9	"	5	5
10	"	7	8
11	"	7	9
12	"	3	5
13	"	6	8
14	"	6	10
22	Non-contaminated	1	3
23	"	4	5
24	"	3	5
25	"	4	4
26	"	2	1
27	"	3	5
28	"	3	4
29	"	4	4
30	"	2	5
31	"	4	5
32	"	1	3
33	"	4	6
34	"	3	3
35	"	2	4
36	"	2	6
37	"	3	3
38	"	1	6
39	"	3	6
40	"	1	4
41	"	1	6
42	"	2	4

[1] Days 0 to 21 of withdrawal.

[2] Control hens received non-contaminated diet days 1 to 14.

[3] Represents number of eggs pooled per day for each treatment.

Table 8. Summary of pooling method for eggs collected days 15 to 21.

Treatment	Hen number	# of eggs laid by each hen from days 15 to 21[1]
Non-contaminated diet	1	5
	2	5
	3	5
	4	6
	5	4
	6	5
	7	6
	8	4
EDB-contaminated diet	1	0
	2	0
	3	0
	4	4
	5	4
	6	2
	7	0
	8	4
	9	2
	10	2
	11	5
	12	5
	13	0
	14	5
	15	5
	16	4

[1] Represents number of eggs pooled per hen over days 15 to 21.

HSGC analysis, samples were thawed and gravimetrically transferred with a disposable pasteur pipet to a sample vial.

HSGC analytical parameters for egg samples were: 1) sample weight = 2.0 g, 2) 20 N H_2SO_4 = 5 ml, 3) digestion time = 15 minutes, 4) equilibration time = 75 minutes, 5) analysis time = 15 minutes, and 6) injection time = 6 seconds.

Concentration of EDB in eggs was inversely predicted from a calibration curve developed in EDB-spiked control egg. Samples were spiked with 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 15, 22.5, 30, 45, 60, 75, 90, 105, or 120 ppb EDB, digested, equilibrated, and injected. Integrated area of the EDB peak was recorded for each dose. An analysis of variance was conducted on areas obtained for doses 0.5 to 7.5 ppb EDB. Areas from doses 0.5 to 4.0 ppb EDB were not significantly different from each other ($P<0.05$) while area at 4.5 ppb EDB was significantly greater than areas from 0.5 to 4.0 ppb EDB ($P<0.05$) (Figure 3). Therefore, the calibration curve with x=ppb EDB and y=integrated area of the EDB peak was calculated from 4.5 to 120 ppb EDB. The prediction equation is $\hat{y}=707x - 509$ with r=.99 (Figure 4). The 95% confidence interval (C.I.) on r extends from .983 to .994, the 95% C.I. on the y-intercept (b_0) is -509±989, and the 95% C.I. on the slope (b_1) is 707±27. Since the C.I. on b_o includes zero, and the C.I. on b_1 does not, one can conclude that the origin is zero and the regression line is not horizontal. 95% C.I.'s on $\hat{u}y/x$ and $\hat{y}/x$ are pictured in Figure 4. There is

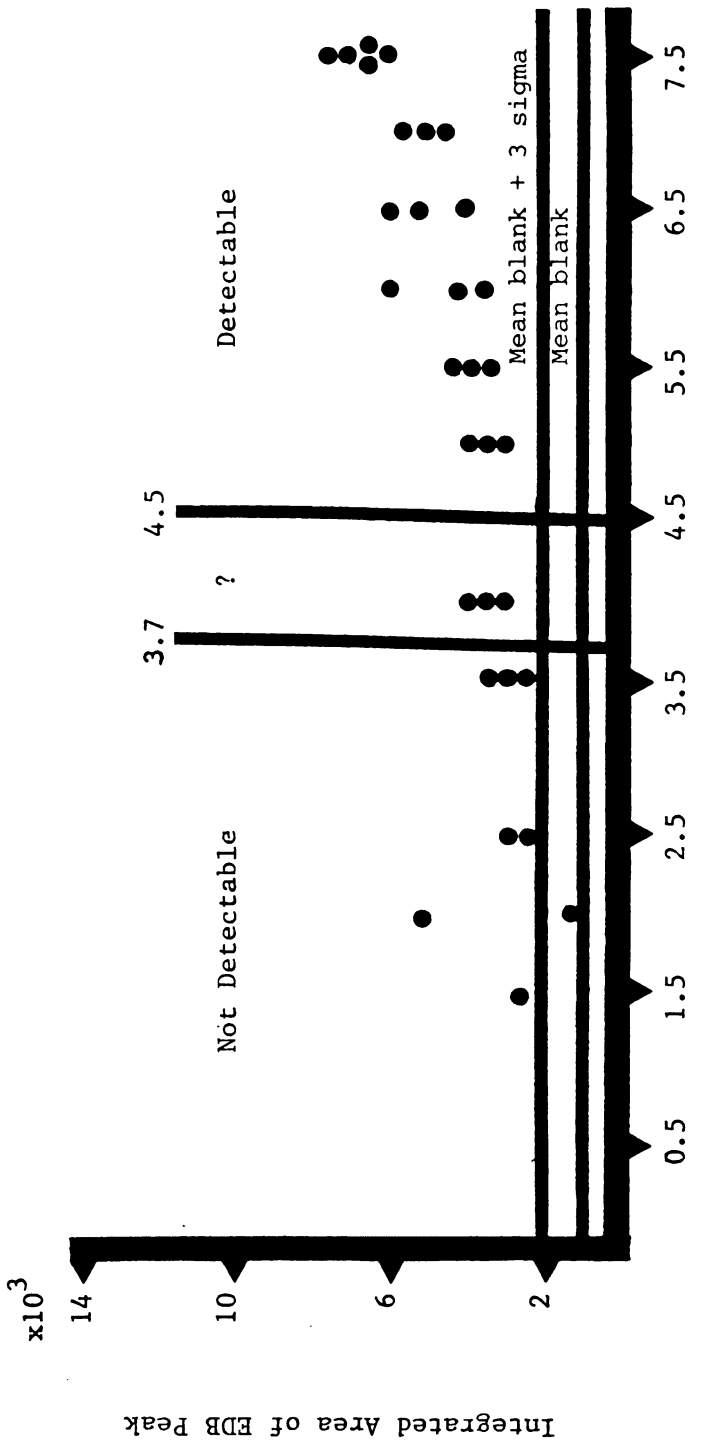

Figure 3. Detection limit of EDB in 5 ml 20N H_2SO_4 + 2g control egg.

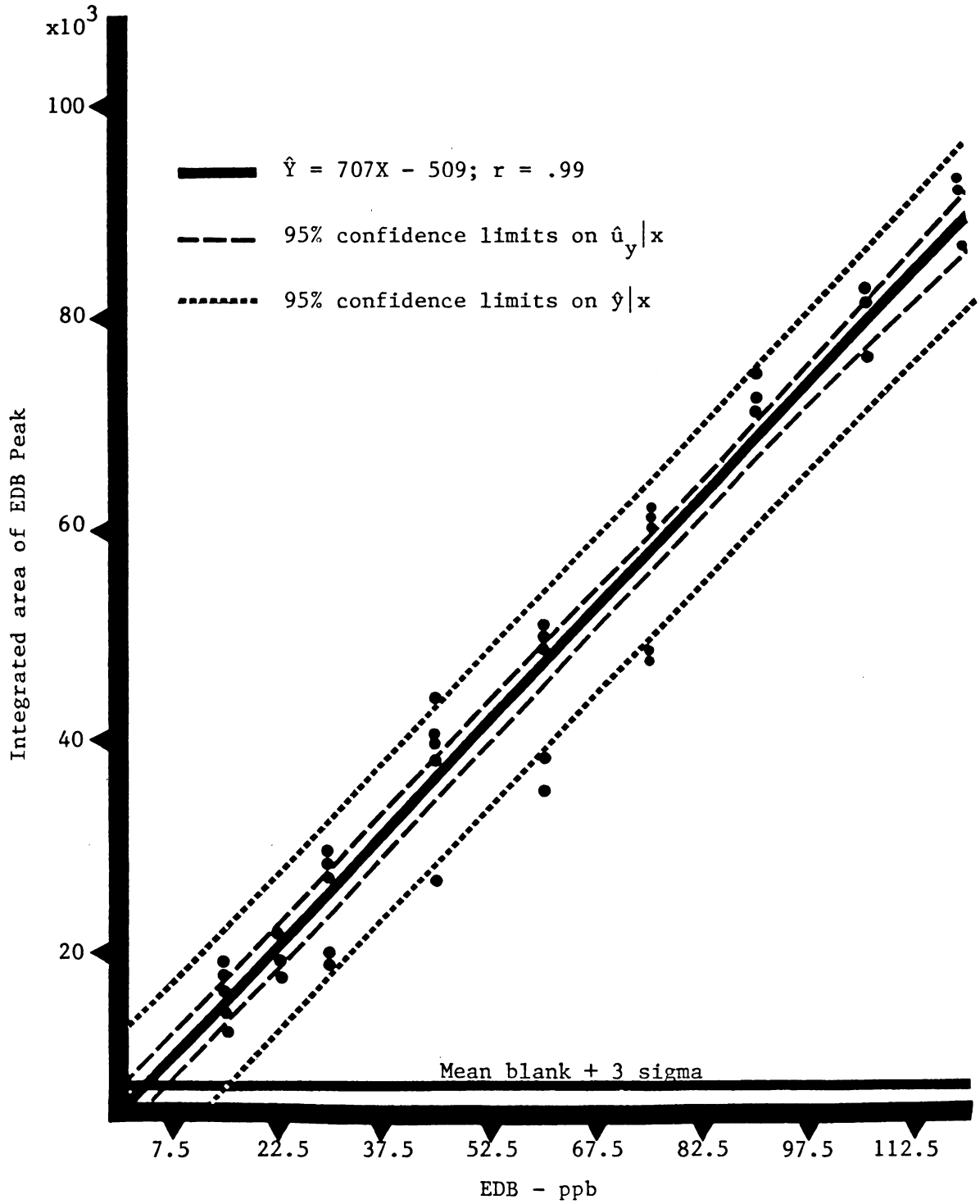

Figure 4. Gas chromatograph dose-response of EDB in 5 ml 20N H_2SO_4 + 2g control egg.

substantial heterogeneity of variances among areas at each dose. Therefore, C.I.'s and predictions based on the preceding prediction equation are biased and are only approximately correct.

The detection limit of EDB in egg was calculated as follows. Mean area + 3 standard deviations was calculated from random fluctuation of 20 non-spiked control egg samples analyzed in the HSGC. That area (2117) is equivalent to a dose of 3.7 ppb EDB. However, since the regression line is not linear until 4.5 ppb EDB, 4.5 ppb EDB is the true detection limit of EDB in egg and values of EDB below 3.7 ppb are not detectable while values of EDB in between 3.7 and 4.5 ppb fall on a portion of the curve in which detectability is uncertain (Figure 3).

See Appendix C for statistical formulas used to calculate the prediction equation and confidence intervals. See Appendix D for egg calibration curve raw data.

F. Analysis of EDB residues in whole body

Chickens chosen for whole body analysis were removed from the freezer, thawed overnight at 4°C, sawed into several small pieces with a Hobart 5212F electric saw, and ground to hamburger consistency (feathers included) in a Hobart 4732 SS electric grinder. Samples were put through the grinder 5 times to obtain a homogeneous sample. Grab samples were removed and frozen in whirl pack bags at -20°C. Prior to HSGC analysis, samples were thawed and gravimetrically transferred with forceps to a sample vial.

HSGC analytical parameters for whole body samples were: 1)

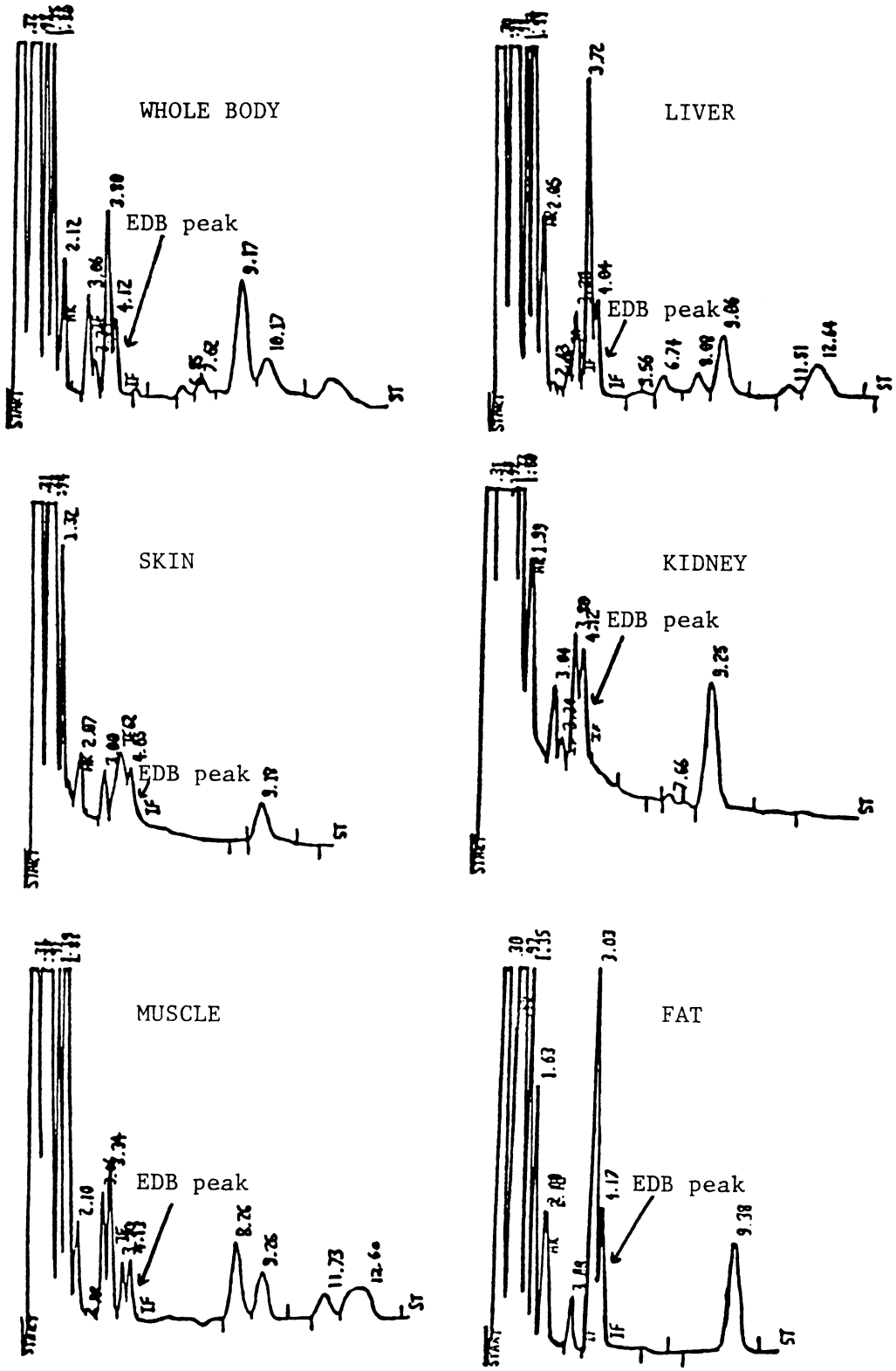

Figure 5. Chromatograms of EDB in tissues.

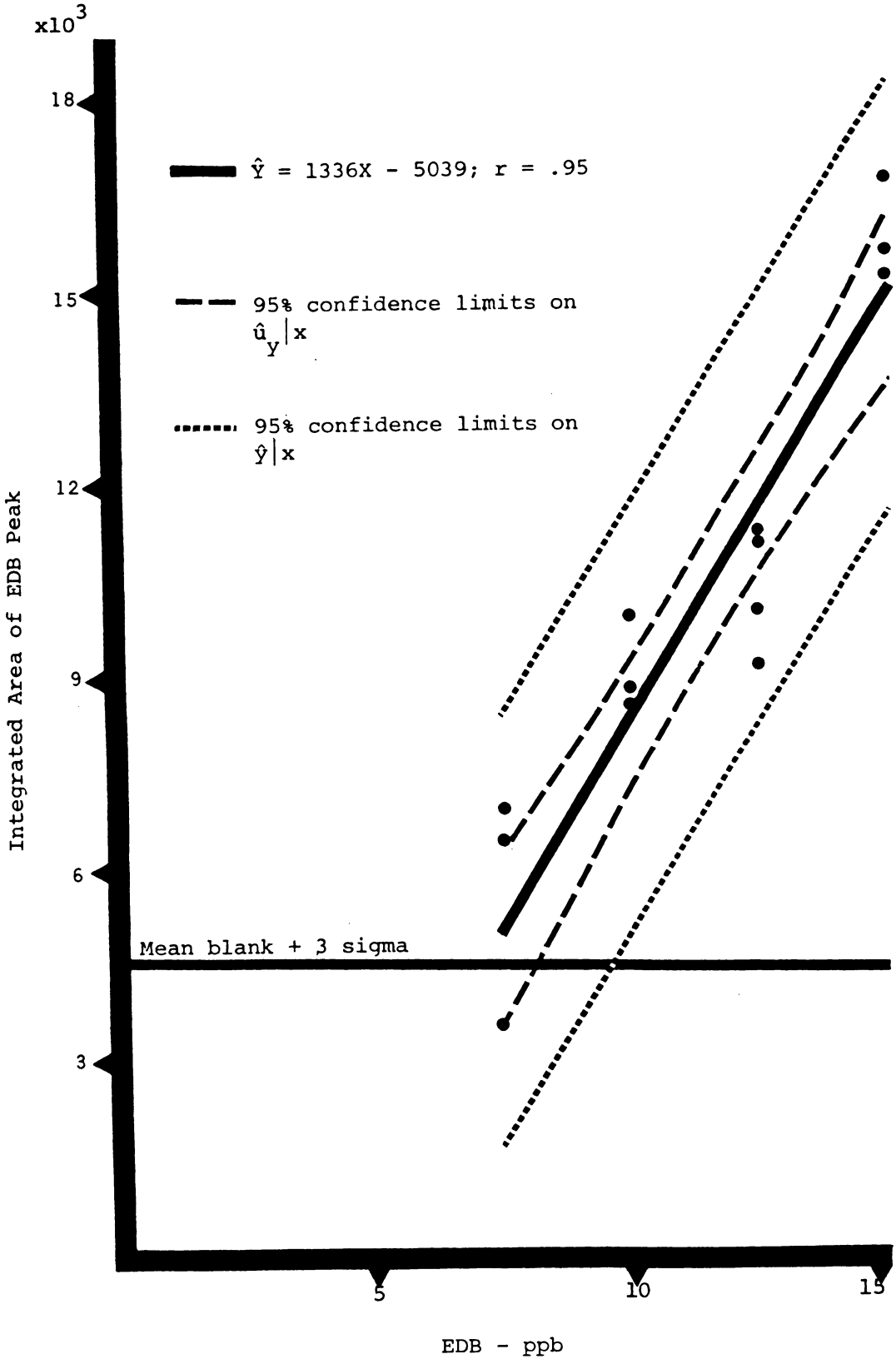

Figure 6. Gas chromatograph dose-response of EDB in 5 ml 20N H_2SO_4 + 2g control whole body.

sample weight = 2.0 g, 2) 20 N H_2SO_4 = 5 ml, 3) digestion time = 30 minutes, 4) equilibration time = 75 minutes, 5) analysis time = 75 minutes, and 6) injection time - 6 seconds. See Figure 5 for a chromatogram.

Concentration of EDB in whole body was inversely predicted from a calibration curve developed in EDB-spiked control samples. Samples were spiked with 7.5, 10, 12.5, or 15 ppb EDB, digested, equilibrated, and injected. Integrated area of the EDB peak was recorded for each dose. The prediction equation with x=ppb EDB and y=integrated area of the EDB peak is $\hat{y}$=1336x - 5039 with r=.95 (Figure 6). The 95% C.I. on r extends from .79 to .98, the 95% C.I. on b_o is -5039±3639, and the 95% C.I. on b_1 is 1336±32. Since the C.I.'s on b_o and b_1 do not include zero, one can conclude that the origin is not zero and that the regression line is not horizontal. 95% C.I.'s on $\hat{u}y/x$ and $\hat{y}/x$ are pictured in Figure 6.

The detection limit of EDB in whole body was calculated as follows. A mean area + 3 standard deviations was calculated from random fluctuation of 20 non-spiked control samples analyzed in the HSGC. That area (4570) is equivalent to a dose of 7.2 ppb EDB and is the detection limit. See Appendix D for whole body calibration curve raw data.

G. Analysis of EDB residues in liver

Liver samples were removed from the freezer, thawed and homogenized individually with a Tekmar SDT Tissumizer. Homogenized samples were stored in whirl pack bags at -20°C. Prior to HSGC

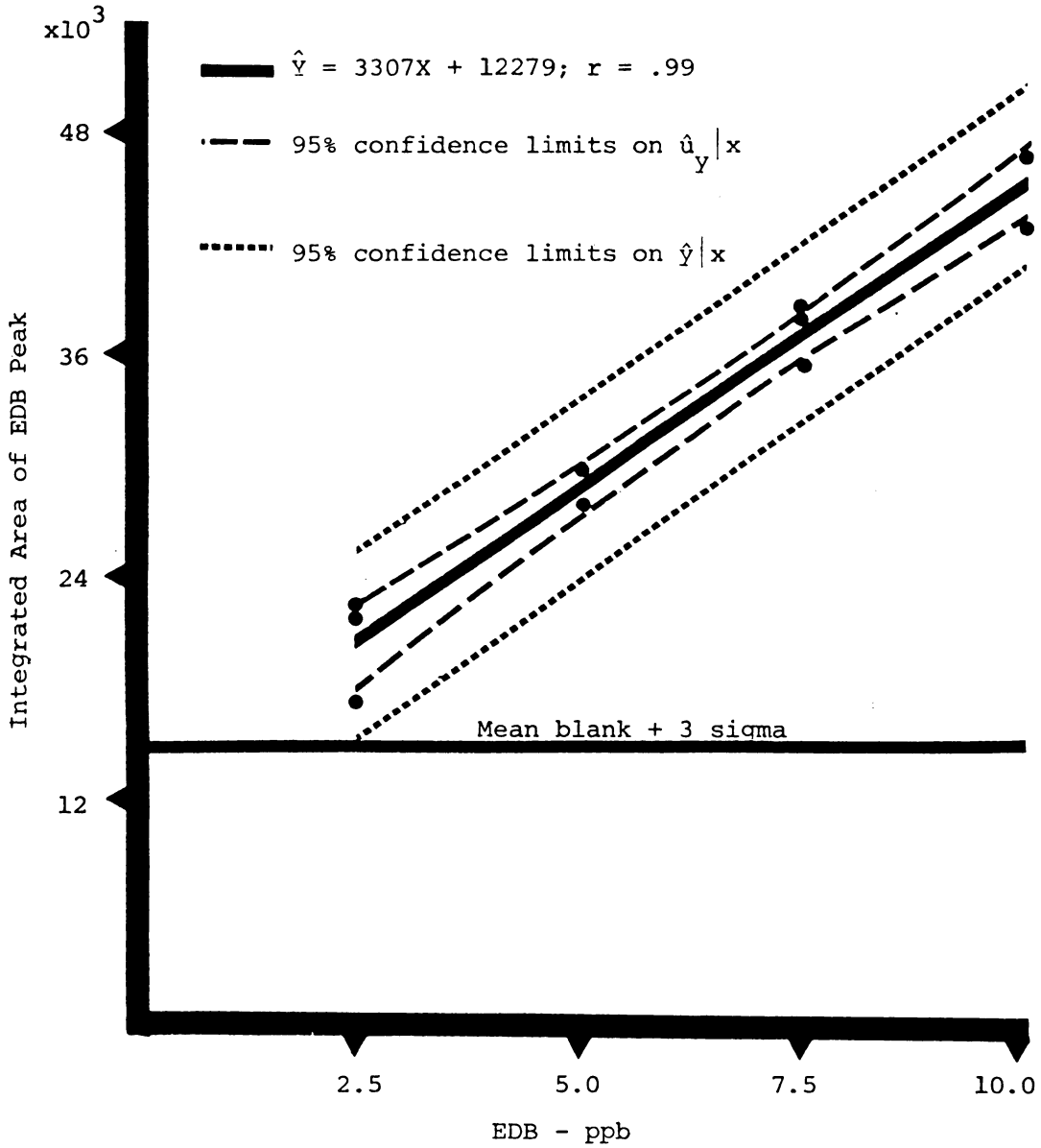

Figure 7. Gas chromatograph dose-response of EDB in 5 ml 20N H_2SO_4 + 2g control liver.

analysis, samples were thawed and gravimetrically transferred with a spatula to a sample vial.

HSGC analytical parameters for liver samples were: 1) sample weight = 2.0 g, 2) 20 N H_2SO_4 = 5 ml, 3) digestion time = 30 minutes, 4) equilibration time = 75 minutes, 5) analysis time = 40 minutes, and 6) injection time = 6 seconds. See Figure 5 for a chromatogram.

Concentration of EDB in liver was inversely predicted from a calibration curve developed in EDB-spiked control samples. Samples were spiked with 2.5, 5.0, 7.5, or 10 ppb EDB, digested, equilibrated, and injected. Integrated area of the EDB peak was recorded for each dose. The prediction equation with x=ppb EDB and y=integrated area of the EDB peak is $\hat{y}$=3307x + 12279 with r=.99 (Figure 7). The 95% C.I. on r extends from .908 to .995, the 95% C.I. on b_o is 12279±3347, and the 95% C.I. on b_1 is 3307±506. Since the C.I.'s on b_o and b_1 do not include zero, one can conclude that the origin is not zero and that the regression line is not horizontal. 95% C.I.'s on $\hat{u}y/x$ and $\hat{y}/x$ are pictured in Figure 7.

The detection limit of EDB in liver was calculated as follows. Mean area + 3 standard deviations was calculated from random fluctuation of 20 non-spiked control samples analyzed in the HSGC. That area (14925) is equivalent to a dose of 0.8 ppb EDB and is the detection limit. See Appendix D for liver calibration curve raw data.

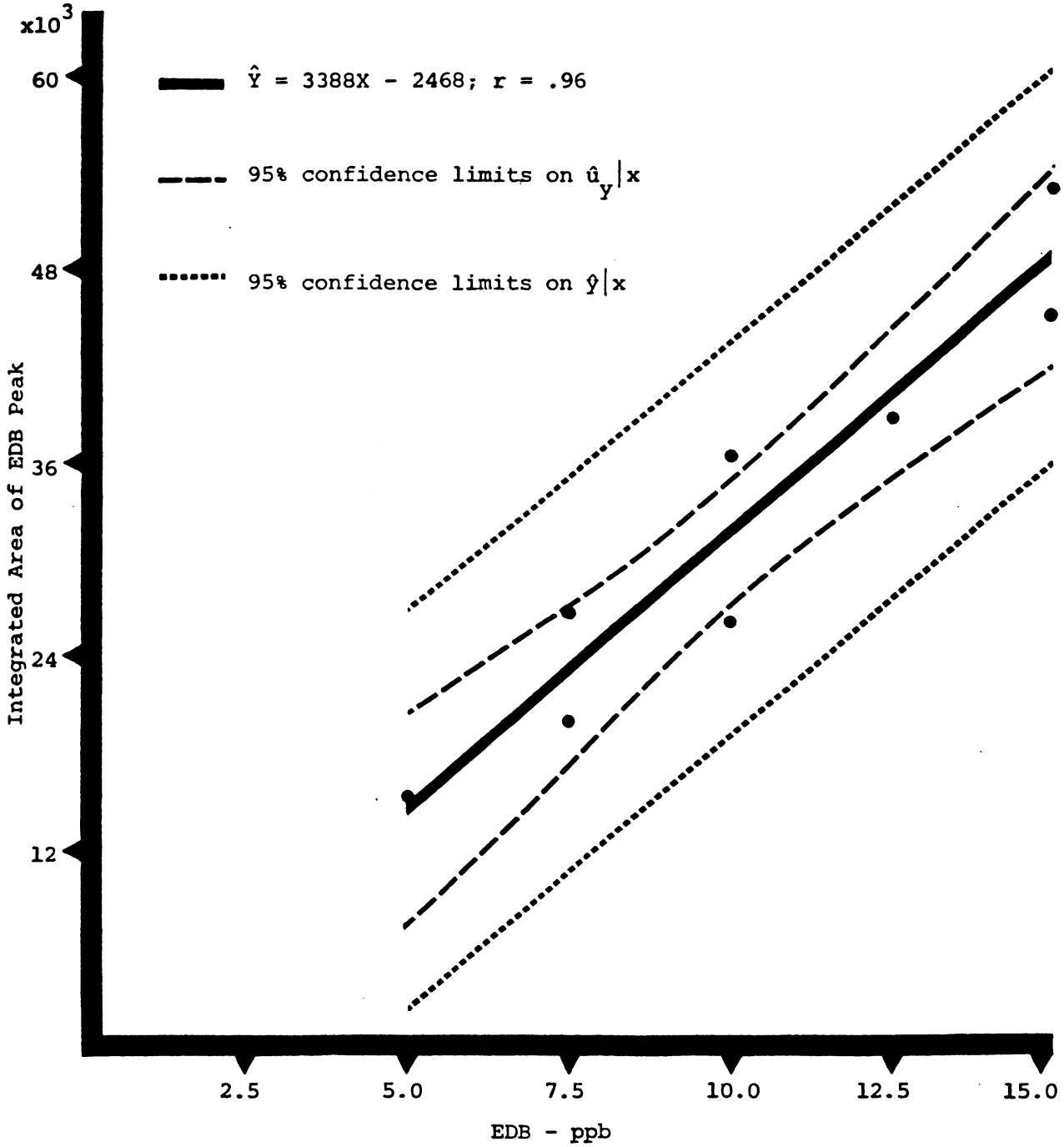

Figure 8. Gas chromatograph dose-response of EDB in 10 ml 20N H_2SO_4 + 1g control kidney.

H. Analysis of EDB residues in kidney

Kidney samples were removed from the freezer, thawed, and gravimetrically transferred with forceps to a sample vial. HSGC analytical parameters for kidney samples were: 1) sample weight = 1.0 g, 2) 20 N H_2SO_4 = 10 ml, 3) digestion time = 30 minutes, 4) equilibration time = 75 minutes, 5) analysis time = 75 minutes, and 6) injection time = 8 seconds. See Figure 5 for a chromatogram.

Concentration of EDB in kidney was inversely predicted from a calibration curve developed in EDB-spiked control samples. Samples were spiked with 5.0, 7.5, 10.0, 12.5, or 15.0 ppb EDB, digested, equilibrated, and injected. Integrated area of the EDB peak was recorded for each dose. The prediction equation with x=ppb EDB and y=integrated area of the EDB peak is $\hat{y}$=3388x - 2468 with r=.96 (Figure 7). The 95% C.I. on r extends from .72 to .99, the 95% C.I. on b_o is -2468±11,047, and the 95% C.I. on b_1 is 3388±1071. Since the C.I. on b_o includes zero while the C.I. on b_1 does not, one can conclude that the origin is zero and that the regression line is not horizontal. 95% C.I.'s on $\hat{u}y/x$ and $\hat{y}/x$ are pictured in Figure 8.

The detection limit of EDB in kidney was calculated as follows. In kidney, the integrator did not recognize separate peaks at retention times 3.80 (unidentified peak) and 4.12 (EDB peak) (Figure 5) at doses below 5.0 ppb EDB. Therefore, 5.0 ppb is the detection limit in kidney. See Appendix D for kidney calibration curve raw data.

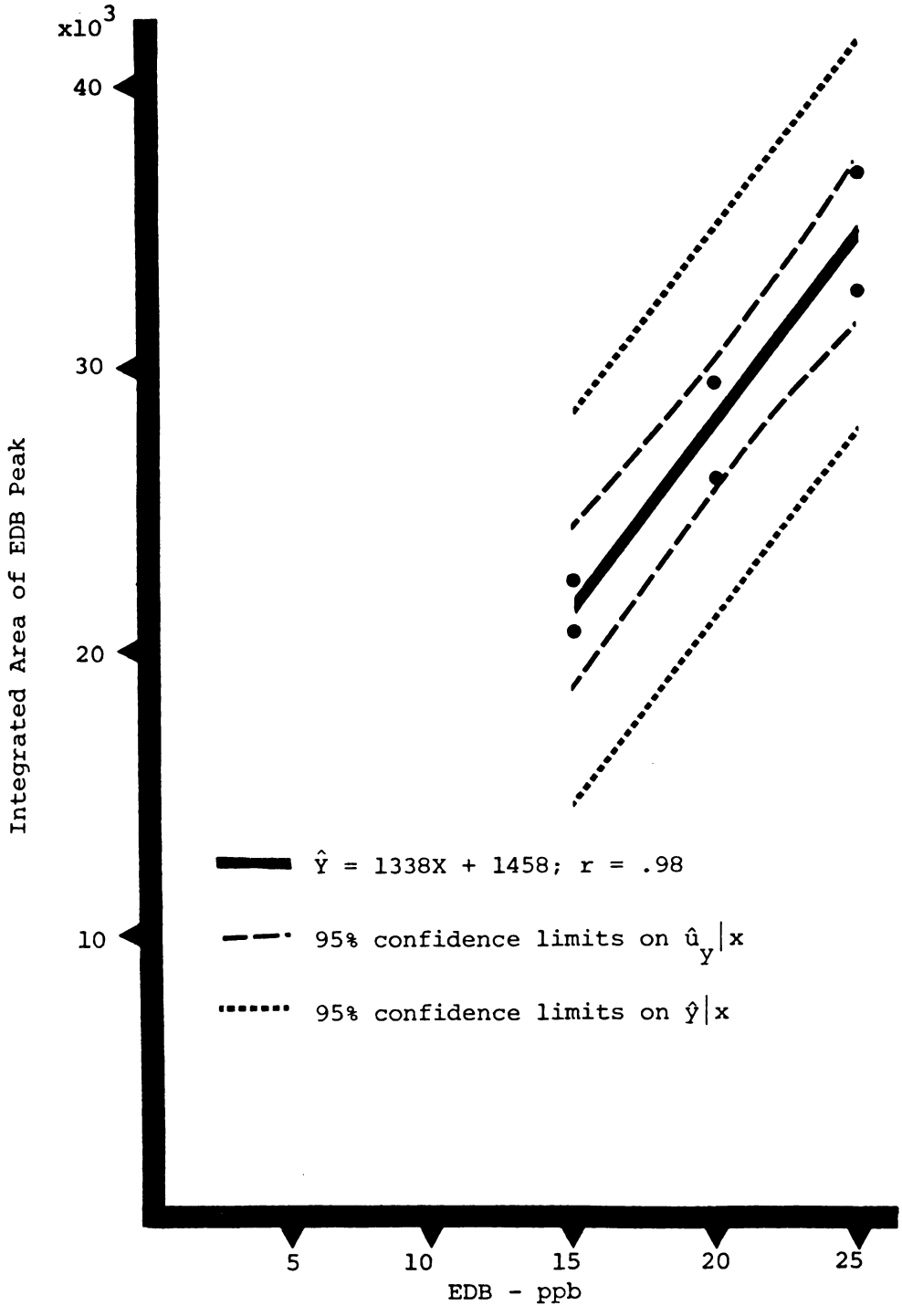

Figure 9. Gas chromatograph dose-response of EDB in 10 ml 20N H_2SO_4 + 0.5g control skin.

I. Analysis of EDB residues in skin

Skin samples were removed from the freezer, thawed, and gravimetrically transferred with forceps to a sample vial. HSGC analytical parameters were: 1) sample weight = 0.5 g, 2) 20 N H_2SO_4 = 10 ml, 3) digestion time = 60 minutes, 4) equilibration time = 120 minutes, 5) analysis time = 15 minutes, and 6) injection time = 8 seconds. See Figure 5 for a chromatogram.

Concentration of EDB in skin was inversely predicted from a calibration curve developed in EDB-spiked control samples. Samples were spiked with 15, 20, or 25 ppb EDB, digested, equilibrated, and injected. Integrated area of the EDB peak was recorded for each dose. The prediction equation with x=ppb EDB and y=integrated area of the EDB peak is $\hat{y}$=1338x + 1458 with r=.98 (Figure 9). The 95% C.I. on r extends from .63 to .99, the 95% C.I. on b_o is 1458±11,928, and the 95% C.I. on b_1 is 1338±584. Since the C.I. on b_o includes zero while the C.I. on b_1 does not, one can conclude that the origin is zero and that the regression line is not horizontal. 95% C.I.'s on $\hat{u}y/x$ and $\hat{y}/x$ are pictured in Figure 9.

The detection limit of EDB in skin was calculated as follows. In skin, the integrator did not recognize separate peaks at retention times 3.62 (unidentified peak) and retention time 4.05 (EDB peak) (Figure 5) at doses below 15 ppb EDB. Therefore, 15 ppb is the detection limit in skin. See Appendix D for skin calibration curve raw data.

J. Analysis of EDB residues in fat

Fat samples were removed from the freezer, thawed, and gravimetrically transferred with a spatula to a sample vial. HSGC analytical parameters were: 1) sample weight = 2.0 g, 2) 20 N H_2SO_4 = 5 ml, 3) digestion time = 15 minutes, 4) equilibration time = 75 minutes, 5) analysis time = 15 minutes, and 6) injection time = 6 seconds. See Figure 5 for a chromatogram.

Concentration of EDB in fat was calculated from standard addition to each fat sample obtained from hens fed EDB-contaminated diet instead of from a calibration curve developed in EDB-spiked control samples because control fat was depleted during determination of fat HSGC analysis parameters. Quantification by standard addition involved spiking known amounts of EDB into samples which contained unknown quantities of EDB. A fat sample for which concentration of EDB was to be determined was partitioned into several 2 g samples. Then 2 of the samples were analyzed in the HSGC as is, i.e. without an EDB spike. The rest of the 2 g samples were analyzed in the HSGC with one of 3 other EDB spikes of varying concentrations. A linear regression equation was calculated from $x_1 = 0$, x_2 = amount of first EDB spike in ppb, x_3 = amount of second EDB spike in ppb, x_4 = amount of third EDB spike in ppb, and y_1 = area from unknown EDB concentration, y_2 = area from unknown EDB concentration plus area due to first spike, y_3 = area from unknown EDB concentration plus area due to second spike and y_4 = area from unknown EDB concentration plus area due

Table 9. Standard addition prediction equations (with confidence intervals) used to calculate concentration of EDB in fat.

Hen #[1]	Prediction equation[2]	95% C.I. on r[3]	95% C.I. on bo[4]	95% CI on b_1[5]
6	$\hat{Y} = 465X + 13610$	$.935 < .99 \leq .999$	$9452 < 13610 < 17768$	$392 < 465 \leq 538$
7	$\hat{Y} = 995X + 102{,}419$	$.940 < .99 \leq .998$	$73145 < 102{,}419 \leq 131093$	$870 < 995 \leq 1120$
8	$\hat{Y} = 697X + 42{,}369$	$.869 < .98 \leq .995$	$10948 < 42{,}369 \leq 73790$	$560 < 697 \leq 834$
3	$\hat{Y} = 368X + 8800$	$.914 < .99 \leq .997$	$6331 < 8800 \leq 11269$	$312 < 368 \leq 424$

[1] Represents code number of the hen from which the fat sample was taken. Samples were from hens killed on day 21 of feeding EDB-contaminated diet (day 0 of withdrawal). Fat samples taken on day 21 of withdrawal did not contain detectable EDB.

[2] X = ppb EDB, Y = integrated area of the EDB peak, $|X\text{-intercept}| = |-bo/b1|$ = ppb EDB in sample.

[3] r = product-moment correlation.

[4] bo = Y-intercept.

[5] b_1 = slope.

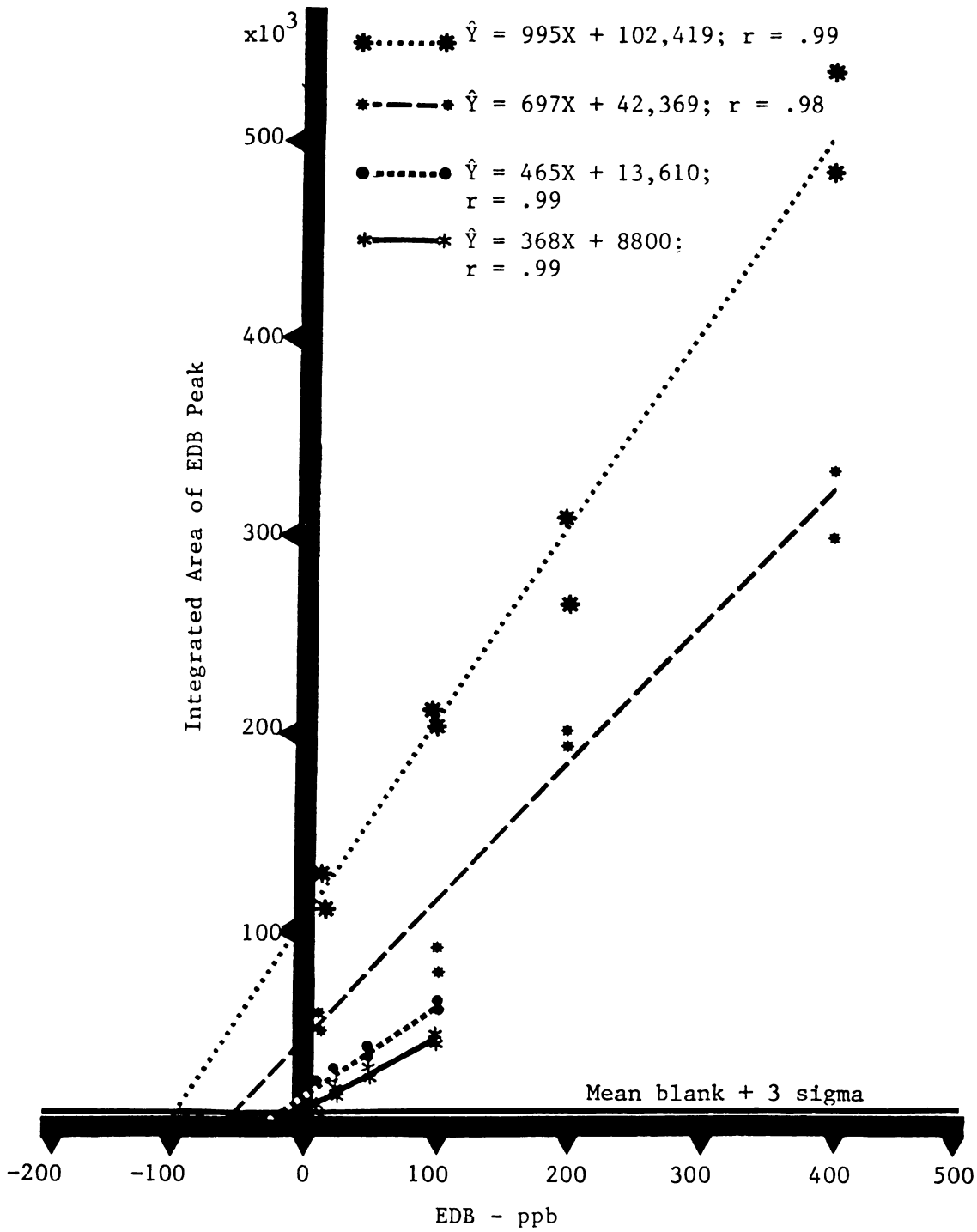

Figure 10. Standard addition dose-response lines for fat.

to third spike. The linear regression parameters (b_o, b_1, r) were calculated as usual, and the concentration of EDB in ppb in unknowns was calculated from |x-intercept| (Harvey 1950).

Four standard addition lines were developed for the 4 fat samples which contained detectable EDB (Figure 10). Two of the fat samples were spiked with 0, 25, 50, or 100 ppb EDB, and 2 of the fat samples were spiked with 0, 100, 200, or 400 ppb EDB. Table 9 contains the standard addition linear regression equations with 95% C.I.'s for r, b_o, and b_1. By comparing the C.I.'s on b_1, it can be seen that only one pair of C.I.'s on b_1 overlap. This indicates that the regression lines are not homogeneous.

The detection limit of EDB cannot be calculated on a ppb basis from standard addition lines because the line representing the area of mean blank + 3 standard deviations crosses the standard addition lines at a point corresponding to a negative x-value (Figure 10). However, the mean area + 3 standard deviations obtained from analyzing 20 non-spiked fat samples in the HSGC was 1705. Any areas below this obtained during fat analysis were considered as random fluctuations and non-detectable.

K. Analysis of EDB residues in muscle

Muscle samples were removed from the freezer, thawed, and gravimetrically transferred with forceps to a sample vial. HSGC analytical parameters were: 1) sample weight = 3.0 g, 2) 20 N H_2SO_4 = 5 ml, 3) digestion time = 30 minutes, 4) equilibration time = 75 minutes, 5) analysis time = 75 minutes, and 6) injection time = 6

Table 10. Standard addition prediction equations (with confidence intervals) used to calculate concentration of EDB in muscle.

Hen #[1]	Prediction equation[2]	95% C.I. on r[3]	95% C.I. on bo[4]	95% C.I. on b_1[5]
6	$\hat{Y} = 4042X + 2351$	$.966 < .99 \leq .999$	$-579 < 2351 \leq 5281$	$3638 < 4042 \leq 4446$
7	$\hat{Y} = 4296X + 2685$	$.978 < .99 \leq .999$	$-397 < 2685 \leq 5767$	$3819 < 4296 \leq 4773$
8	$\hat{Y} = 4459X + 1439$	$.940 < .99 \leq .999$	$-5346 < 1439 \leq 8224$	$3499 < 4459 \leq 5419$
3	$\hat{Y} = 4174X + 868$	$.988 < .99 \leq .999$	$-596 < 868 \leq 2332$	$3947 < 4174 \leq 4401$

[1] Represents code number of the hen from which right breast muscle was taken. Samples were from hens killed on day 21 of feeding EDB-contaminated diet (day 0 of withdrawal). Muscle samples taken on day 21 of withdrawal did not contain detectable EDB.

[2] X = ppb EDB, Y = integrated area of the EDB peak, $|X\text{-intercept}| = |-bo/b1| =$ ppb EDB in sample.

[3] r = product-momeht correlation.

[4] bo = Y-intercept.

[5] b_1 = slope.

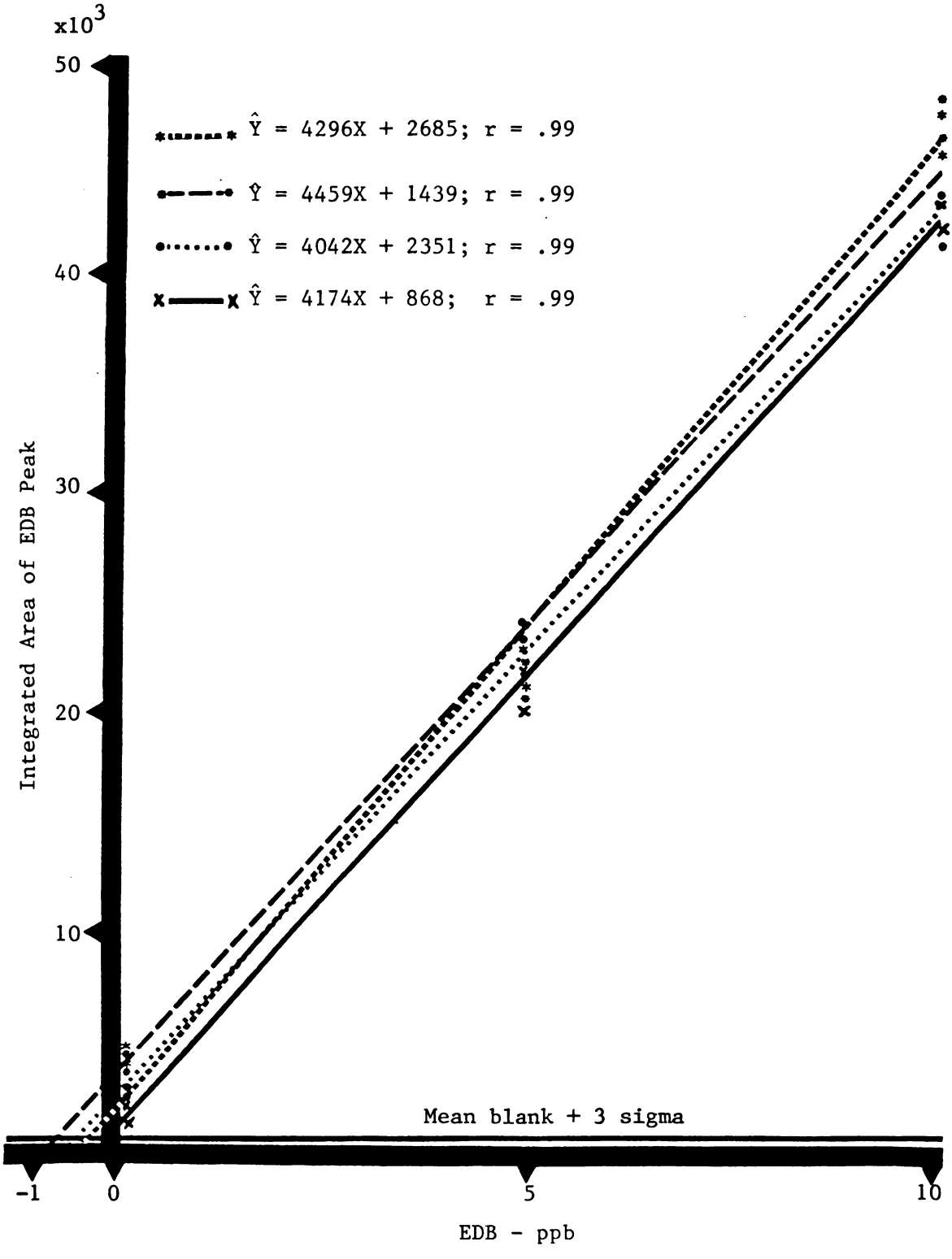

Figure 11. Standard addition dose-response lines for muscle.

seconds. See Figure 5 for a chromatogram.

Concentration of EDB in muscle was calculated from standard addition to muscle samples obtained from hens fed EDB-contaminated diet instead of from a calibration curve in control samples because control muscle was depleted during determination of muscle HSGC analysis parameters. Four standard addition lines were developed (following the general standard addition procedure outlined in the fat analysis section) for the 4 muscle samples which contained detectable EDB (Figure 11). Table 10 contains the standard addition linear regression equations with 95% C.I.'s for r, b_o and b_1. By comparing the C.I.'s on b_1, it can be seen that all of the C.I.'s on b_1 overlap indicating homogeneity of regression among the muscle samples obtained from different hens.

The detection limit of EDB in muscle could not be calculated on a ppb basis for the same reasons as outlined under fat analysis. However, the mean area + 3 standard deviations obtained from analyzing 20 non-spiked muscle samples in the HSGC was 696. Any areas below this obtained during muscle analysis were considered as random fluctuations and non-detectable.

L. Analysis of EDB residues in flour

Subsamples of flour were taken just prior to blending of experimental diets, placed in whirl pack bags, and stored at -20°C. Prior to HSGC analysis, samples were warmed to room temperature and gravimetrically transferred with a spatula to a sample vial. HSGC analytical parameters were: 1) sample weight =

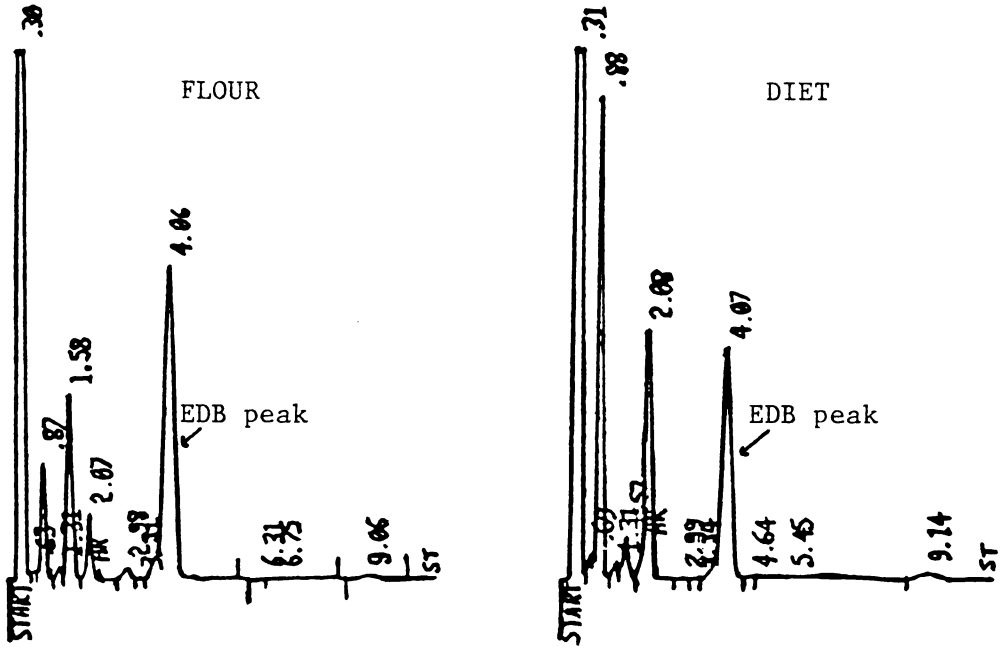

Figure 12. Chromatograms of EDB in diet and flour.

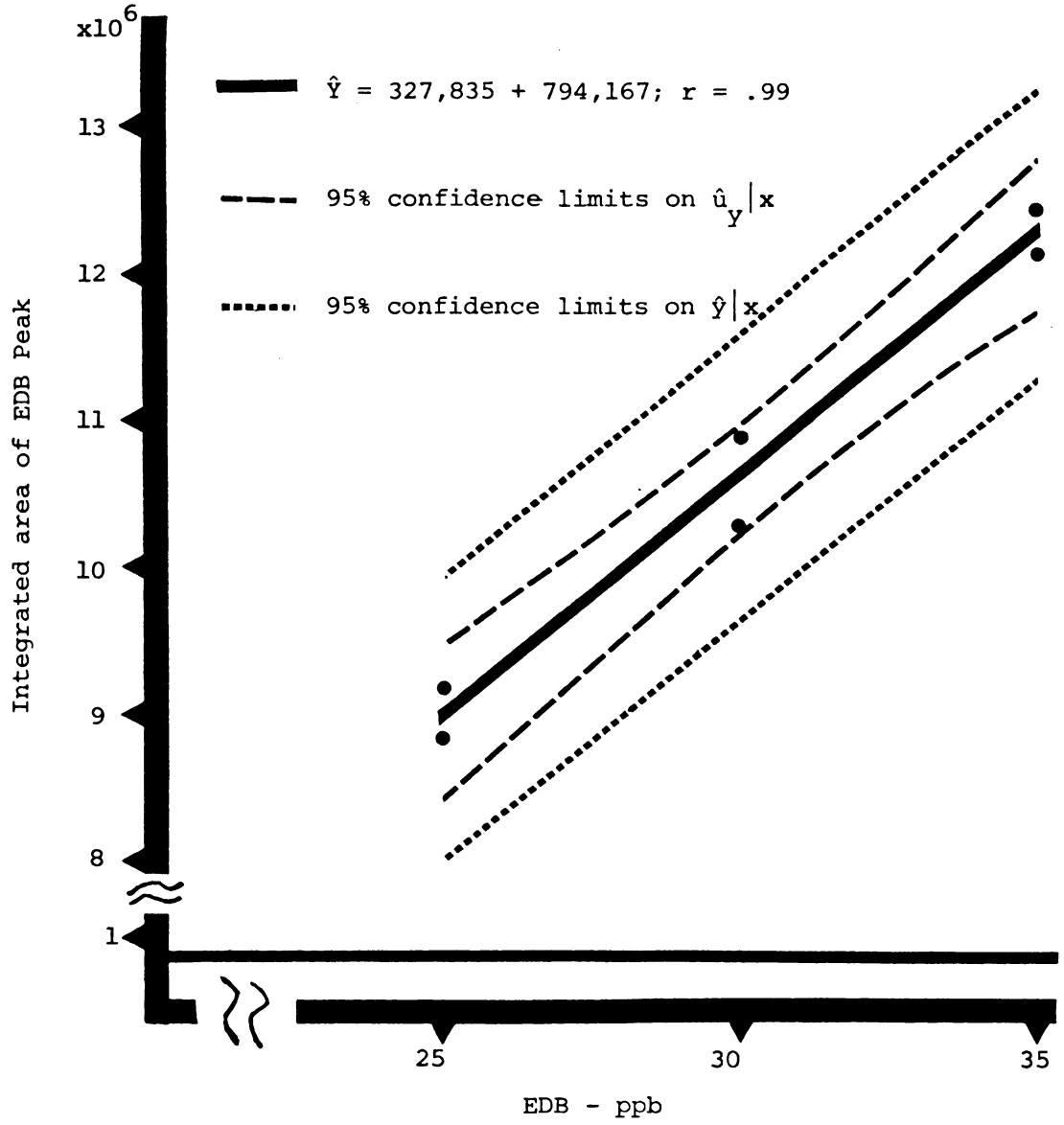

Figure 13. Gas chromatograph dose-response of EDB in 10 ml 20N H_2SO_4 + 0.1g control flour.

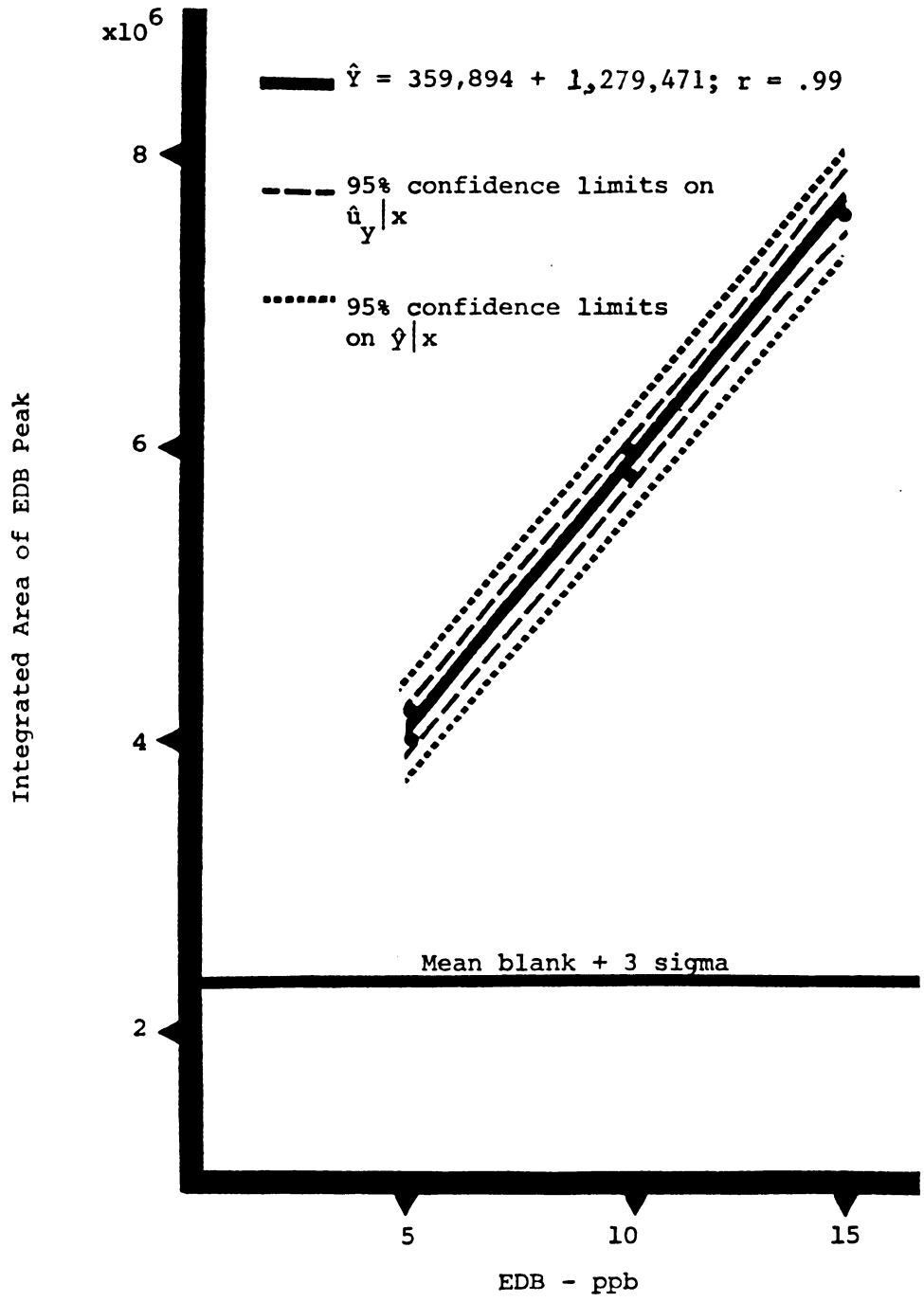

Figure 14. Gas chromatograph dose-response of EDB in 10 ml 20N H_2SO_4 + 0.1g control diet.

0.1 g, 2) 20N H_2SO_4 = 10 ml, 3) digestion time = 60 minutes, 4) equilibration time = 75 minutes, 5) analysis time = 20 minutes, and 6) injection time = 8 seconds. See Figure 12 for a chromatogram.

Concentration of EDB in flour was inversely predicted from a calibration curve developed in EDB-spiked control samples. Samples were spiked with 25, 30, or 35 ppm EDB, digested, equilibrated, and injected. Integrated area of the EDB peak was recorded for each dose. The prediction equation with x=ppm EDB and y=integrated area of the EDB peak is $\hat{y}$=327,835x + 794,167 with r=.99 (Figure 13). The 95% C.I. on r extends from .841 to .998, the 95% C.I. on b_o is 794,167±2,510,249, and the 95% C.I. on b_1 is 327,835±82,911. Since the C.I. on b_o includes zero while the C.I. on b_1 does not, one can conclude that the origin is zero and that the regression line is not horizontal. 95% C.I.'s on $\hat{u}y/x$ and $\hat{y}/x$ are pictured in Figure 13.

The detection limit of EDB in flour was calculated as follows. Mean area + 3 standard deviations was calculated from random fluctuation of 20 non-spiked control samples analyzed in the HSGC. That area (858,235) is equivalent to a dose of .19 ppm EDB and is the detection limit. See Appendix D for flour calibration curve raw data.

M. Analysis of EDB residues in diet

Subsamples of diet were taken just after blending of experimental diets was completed. Samples were stored in whirl pack bags at -20°C. Prior to HSGC analysis, samples were warmed to

Table 11. Summary of prediction equations and detection limits used to calculate concentration of EDB in eggs, whole body, liver, kidney, skin, diet, and flour.

Sample	Prediction equation[1]	r^2	Detection limit[3]-ppb
Egg	$\hat{Y} = 707X - 509$	.99	4.5
Whole body	$\hat{Y} = 1336X - 5039$	.95	7.2
Liver	$\hat{Y} = 3307X - 12279$	.99	0.8
Kidney	$\hat{Y} = 3388X - 2468$	.96	5.0
Skin	$\hat{Y} = 1338X + 1458$	.98	15.0
Diet	$\hat{Y} = 359{,}894X + 1{,}279{,}471$	.99	230.0
Flour	$\hat{Y} = 327{,}835X + 794{,}167$	.99	190.0

[1] X = ppb EDB, Y = integrated area of the EDB peak.

[2] r = product-moment correlation.

[3] Calculated from random fluctuation of non-spiked control samples for egg, whole body, liver, diet, and flour. Calculated from smallest dose which could be integrated in skin and kidney.

room temperature and gravimetrically transferred with a spatula to a sample vial. HSGC analytical parameters were: 1) sample weight = 0.1 g, 2) 20 N H_2SO_4 = 10 ml, 3) digestion time = 60 minutes, 4) equilibration time = 75 minutes, 5) analysis time = 20 minutes, and 6) injection time = 8 seconds. See Figure 12 for a chromatogram.

Concentration of EDB in diet was inversely predicted from a calibration curve developed in EDB-spiked control samples. Samples were spiked with 5, 10 or 15 ppm EDB, digested, equilibrated, and injected. Integrated area of the EDB peak was recorded for each dose. The prediction equation with x=ppm EDB and y=integrated area of the EDB peak is $\hat{y}$=359,894x + 1,279,471 with r=.99 (Figure 14). The 95% C.I. on r extends from .985 to .995, the 95% C.I. on b_o is 1,279,471 ± 300,820, and the 95% C.I. on b_1 is 359,894±30,863. Since the C.I.'s on b_o and b_1 do not include zero, one can conclude that the origin is not zero and that the regression line is not horizontal. 95% C.I.'s on $\hat{u}y/x$ and $\hat{y}/x$ are pictured in Figure 14.

The detection limit of EDB in diet was calculated as follows. Mean area + 3 standard deviations was calculated from random fluctuation of 20 non-spiked control samples analyzed in the HSGC. That area (1,362,978) is equivalent to a dose of .23 ppm EDB and is the detection limit. See Appendix D for diet calibration curve raw data.

See Table 11 for a summary of prediction equations and detection limits in egg, tissues, diet, and flour.

RESULTS

I. Feed Consumption, Body Weights, Egg Production, and Egg Weights

Because it was necessary to house EDB and control hens in different environments, this study could not be designed to determine if feeding EDB-contaminated diet for 21 days would have an effect on feed consumption, body weights, egg production, or egg weights. Therefore, although data on those parameters were collected (see Appendix B), statistical analysis of it is not valid.

II. Residues of EDB in Diet and EDB Intake

EDB loss occurs during mixing, storage, and aeration of diet in feeding troughs (Fuller and Morris 1963 and Morris and Fuller 1963). These losses must be quantified if an accurate estimate of the concentration of EDB in diet at the time of ingestion is desired. During this study, flour samples were obtained just prior to blending of experimental diets while dietary samples were obtained after blending was completed. EDB-contaminated flour (EF) contained 31.1 ppm EDB. This level of EDB is much higher than has been typically found in flour samples obtained from EDB-contaminated grain implying that the flour was directly fumigated. EDB-contaminated diet (ED) contained 6.7 ppm EDB. Since EF constituted 55.7% of ED, theoretically, ED should have

contained 17.3 ppm EDB. Thus, when EF was blended into diet, it retained 38.7% and lost 61.3% of its EDB residues. Most of this loss was probably due to evaporation of EDB. Losses during storage and from aeration in feeding troughs throughout the course of this study cannot be quantified because dietary samples were not obtained during those periods. It can be assumed that some losses did occur so that actual concentration of EDB in diet at the time of ingestion by hens was less than 6.7 ppm.

EDB hens consumed a total of 2.19 kg of ED per hen over the 21 day period during which ED was fed. Assuming that EDB content in diet (6.7 ppm) was not reduced during that time, this is equivalent to a total intake of 14.7 mg of EDB per hen. Since some evaporation of EDB from ED probably did occur, actual EDB intake per hen would have been somewhat less than 14.7 mg.

III. Residues of EDB in Egg, Whole Body, and Tissues

Residues of EDB were not detected in any control egg, control whole body, or control tissue samples. See Table 15 for detection limits. Egg samples obtained from EDB hens were homogenized with a Waring blender prior to analysis. EDB residues in egg could have been reduced during this process via evaporation. Thus, residues reported below are biased to the degree that residues may have declined during homogenization.

The average concentration (ppb) and burden (ng) of EDB in egg for each day from days 1 to 21 of residue build-up (RB), i.e. days

Table 12. Residues of EDB in eggs obtained from EDB hens on days 1 to 14 of residue buildup and days 1 to 21 of withdrawal.

Treatment	Day	EDB-ppb[1]	Average egg weight-grams[2]	Nanograms of EDB per egg[3]
Residue buildup	1	ND[4]	--	---
	2	ND	--	---
	3	9.8 ± 10.8	53.7	524 ± 582
	4	13.5 ± 10.8	53.2	719 ± 576
	5	16.6 ± 10.8	51.3	854 ± 555
	6	21.0 ± 10.8	52.5	1100 ± 567
	7	22.0 ± 10.8	52.2	1147 ± 564
	8	26.4 ± 10.8	52.9	1397 ± 571
	9	25.4 ± 10.8	53.1	1349 ± 573
	10	23.7 ± 10.8	54.4	1290 ± 588
	11	24.6 ± 10.8	54.3	1335 ± 586
	12	28.9 ± 10.8	55.1	1591 ± 595
	13	25.3 ± 10.8	53.9	1364 ± 582
	14	25.3 ± 10.8	54.6	1381 ± 589
Withdrawal	1	17.5 ± 10.8	54.7	955 ± 591
	2	16.7 ± 10.8	53.1	886 ± 574
	3	11.9 ± 10.8	53.4	638 ± 578
	4	7.6 ± 10.8	52.0	395 ± 564
	5	8.0 ± 10.8	52.2	417 ± 566
	6-21	ND	--	---

[1] Values represent concentration of EDB (with a 95% confidence interval) in egg sample pooled from all eggs laid by EDB hens on specified day.

[2] Represents average weight of egg contents (yolk + albumen) of eggs used for residue analysis on each day. Weight of egg contents was calculated by subtracting shell weight from total weight.

[3] Nanograms of EDB per egg (with a 95% confidence interval) was calculated by multiplying concentration of EDB in egg times average egg weight.

[4] ND; detection limit = 4.5 ppb EDB.

Table 13. Residues of EDB in eggs obtained from EDB hens days 15 to 21 of residue buildup.

Treatment	Hen #	EDB-ppb[1]	Average egg weight-grams[2]	Nanograms of EDB per egg[3]
Residue buildup	1	NE[4]	--	---
	2	NE	--	---
	3	NE	--	---
	4	25.3 ± 10.8	52.1	1318 ± 563
	5	23.8 ± 10.8	54.6	1299 ± 590
	6	23.0 ± 10.8	52.1	1198 ± 563
	7	NE	--	---
	8	32.7 ± 10.8	51.8	1694 ± 559
	9	27.6 ± 10.8	54.7	1510 ± 591
	10	22.9 ± 10.8	57.5	1317 ± 621
	11	23.9 ± 10.8	55.6	1329 ± 600
	12	35.1 ± 10.8	54.7	1920 ± 591
	13	NE	--	---
	14	36.5 ± 10.8	55.0	2008 ± 594
	15	25.5 ± 10.8	51.5	1313 ± 556
	16	29.5 ± 10.8	56.4	1664 ± 609
Mean ± 95% C.I.[5]		27.8 ± 3.3	--	1506 ± 185

[1] Values represent concentration of EDB (with a 95% confidence interval) in egg sample pooled from all eggs laid by each hen over days 15 to 21.

[2] Represents average weight of egg contents (yolk + albumen) of eggs used for residue analysis for each hen. Weight of egg contents was calculated by subtracting shell weight from total weight.

[3] Nanograms of EDB per egg (with a 95% confidence interval) was calculated by multiplying concentration of EDB in egg times average egg weight.

[4] NE = no. eggs laid by that hen during days 15 to 21.

[5] C.I. = confidence interval.

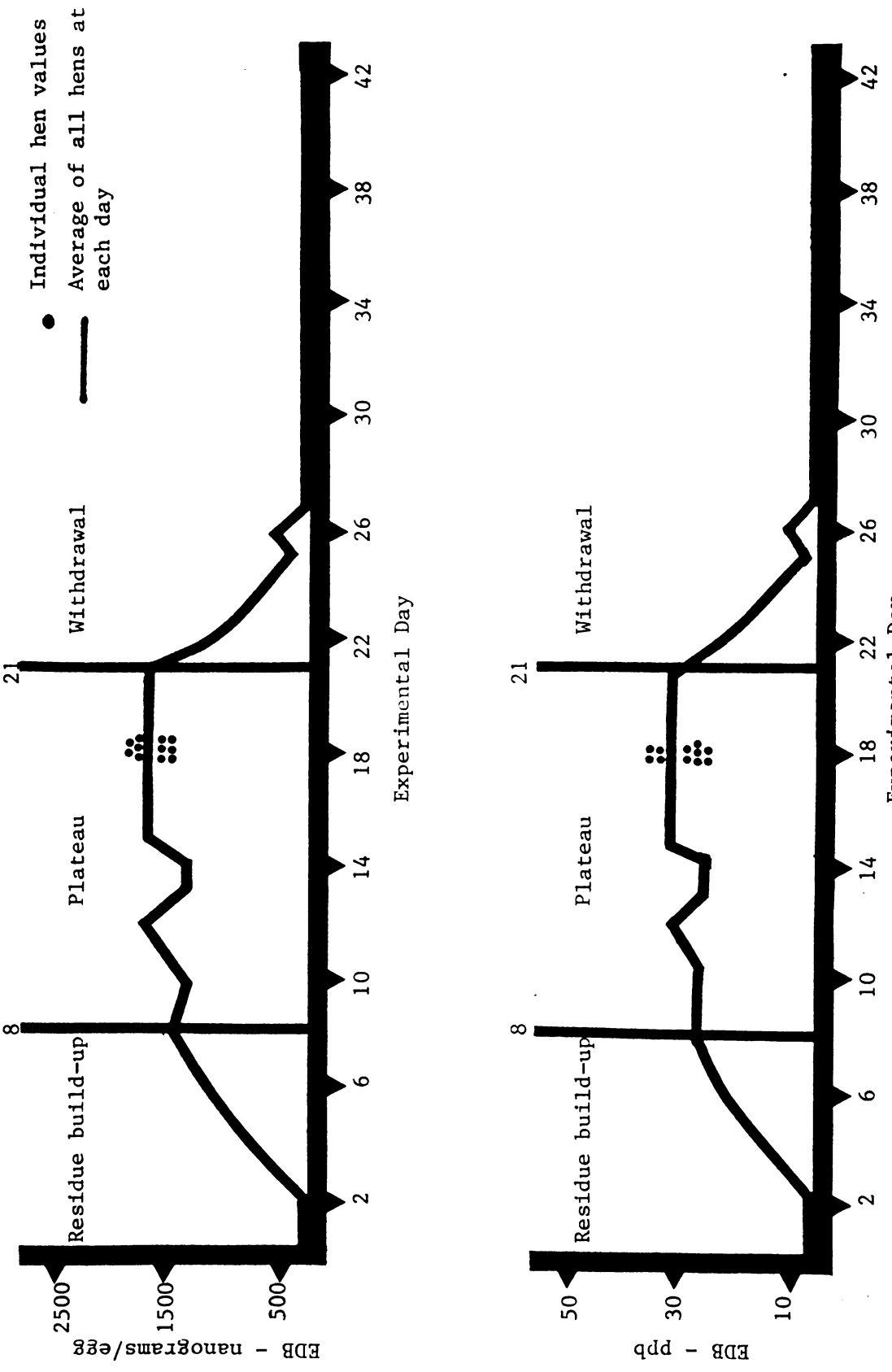

Figure 15. Residues of EDB in eggs obtained from EDB hens during residue build-up, plateau, and withdrawal.

Table 14. Residues of EDB in scrambled eggs before and after frying egg samples obtained from 4 EDB hens during days 15 to 21 of residue buildup.

Hen #	Concentration before frying[1]	Concentration after frying[1]	% of residues retained	% of residues lost
9	27.6	17.3	62.7	37.3
10	22.9	13.9	60.7	39.3
11	23.9	13.7	57.3	42.7
12	35.1	19.7	56.1	43.9
Mean ± SE[2]	27.4 ± 2.8	16.2 ± 1.4	59.2 ± 1.5	40.8 ± 1.5

[1] ppb EDB.
[2] SE = standard error of the mean.

Table 15. Residues of EDB in egg, whole body, and tissues of EDB hens on day 0 of withdrawal.

Egg[1]	Whole body[2]	Fat[3]	Muscle[4]	Liver[5]	Kidney[6]	Skin[7]
-[8]	10.3(NL)[9]	29	0.58	ND[10]	ND	ND
-	9.9(NL)	103(NL)	0.63(NL)	ND	ND	ND
-	10.8	61	0.32	ND	ND	ND
-	12.1	24(NL)	0.21(NL)	ND	ND	ND
27.8 ± 3.3[11]	10.8 ± 1.5[11]	54 ± 58[11]	0.44 ± 0.32[11]	--	--	--

[1] Detection limit is 4.5 ppb EDB.
[2] Detection limit is 7.2 ppb EDB.
[3] Detection limit is equivalent to an area of 1405.
[4] Detection limit is equivalent to an area of 696.
[5] Detection limit is 0.8 ppb EDB.
[6] Detection limit is 5.0 ppb EDB.
[7] Detection limit is 15.0 ppb EDB.
[8] See Table 13 for individual values for egg.
[9] NL indicates hen was not laying for at least 7 days at the time the sample was obtained.
[10] Not detected.
[11] Represents mean ± 95% confidence interval.

1 to 21 of feeding EDB-contaminated diet (ED), and days 1 to 21 of withdrawal are given in Table 12. The average concentration (ppb) and burden (ng) of EDB in eggs obtained from EDB hens days 15 to 21 of residue build-up are given in Table 13. The average concentration of EDB found in egg days 15 to 21 was 27.8 ppb (1506 ng EDB per egg) with the 95% confidence interval (C.I.) ranging from 24.5 to 31.1 ppb (1321 to 1691 ng EDB per egg). Since this range is the maximum concentration of EDB expected in egg, it is apparent that maximum concentration of EDB in egg was reached by day 8 of feeding ED. EDB was first detected in eggs at 9.8 ppb (524 ng) on day 3 of feeding ED. Residues increased linearly in egg from day 3 to day 8 of feeding ED at which time plateau concentration was reached. Concentration of EDB in egg dropped 37% the first day after withdrawal of ED and then decreased linearly until EDB was no longer detectable in egg by day 6 of withdrawal.

The concentration of EDB in four egg samples obtained from EDB hens during days 15 to 21 of residue build-up was determined in scrambled eggs before and after frying (Table 14). On average, frying reduced residues 40.8%.

Concentration of EDB in whole body and tissues is presented in Table 15. On day 0 of withdrawal, whole body, abdominal fat, and breast muscle obtained from EDB hens contained on average (with the 95% C.I.) 10.8±1.5, 54±58, and 0.44±0.32 ppb EDB, respectively. The C.I.'s for fat and muscle are large because of high variability of response among hens. This variability is not

Table 16. Total residue of EDB (ng) deposited into eggs obtained days 1 to 21 of residue buildup from the 4 EDB hens used for whole body analysis on day 0 of withdrawal.

Hen #	Days an egg was laid[1]	Nanograms EDB per egg[2]
1	3	524
	5	854
	7	1147
Total EDB deposited in egg		2525 (or 2.5 µg)
2	3	524
	4	719
	7	1147
Total EDB deposited in egg		2390 (or 2.4 µg)
4	3	524
	4	719
	6	1100
	7	1147
	8	1397
	10	1290
	11	1335
	13	1364
	14	1381
	15	1318
	17	1318
	19	1318
	20	1318
Total EDB deposited in egg		15,529 (or 15.5 µg)
5	3	524
	4	719
	6	1100
	7	1147
	9	1349
	10	1290
	11	1335
	13	1364
	14	1381
	15	1299
	17	1299
	19	1299
	21	1299
Total EDB deposited in egg		15,405 (or 15.4 µg)

[1] Represents days from 1 to 21 of residue buildup.

[2] Values were obtained from Table 12.

Table 17. Distribution of EDB into whole body and egg during residue buildup.

Hen #	EDB intake (µg)[1]	Whole body residue (ppb)[2]	Body weight (g)[3]	Whole body residues[4] µg per bird	% of intake	% of total	Egg residues[5] µg per bird	% of intake	% of total	Total residues µg per bird[6]	% of intake[7]
1	14,700	10.3	1712	17.6	0.12	87.6	2.5(NL)[8]	0.2	12.4	20.1	0.14
2	14,700	9.9	2030	20.1	0.13	89.3	2.4(NL)	0.02	10.7	22.5	0.15
3	14,700	12.1	2040	24.7	0.17	61.4	15.5	0.10	38.6	40.2	0.27
5	14,700	10.8	1856	20.0	0.14	56.5	15.4	0.10	43.5	35.4	0.24
Mean ± 95% C.I.[9]	--	10.8 ± 1.5	--	22.3 ± 21.1[10]	0.15 ± 0.03[10]	58.9 ± 22.0[10]	15.5 ± 0.45[10]	0.11 ± 0.0[10]	41.1 ± 22.0[10]	37.8 ± 21.6[10]	0.26 ± 0.13[10]

[1] Represents total amount of EDB consumed per hen.

[2] Represents concentration of EDB in whole body after 21 days of consuming EDB-contaminated diet, i.e., concentration at day 0 of withdrawal.

[3] Represents body weight at day 21 of residue buildup (day 0 of withdrawal).

[4] µg per bird = (concentration in ppb X weight in grams)/1000; % of intake = (µg per bird/14,700) X 100; % of total = (µg per bird/total µg per bird) X 100.

[5] See Table 16 for method of determining total µg of EDB deposited into egg during residue buildup. % of intake and % of total are calculated as for whole body.

[6] Total µg per bird = whole body µg per bird + egg µg per bird; represents total amount of EDB deposited into tissues and egg.

[7] Represents (total µg EDB per bird/14,700) X 100.

[8] NL indicates that the hen stopped laying after the seventh day of residue buildup.

[9] C.I. = confidence interval.

[10] Means and C.I.'s are calculated only for hens which were laying.

related to the hen's state of production because for both fat and muscle, the highest and lowest responses occurred in non-laying hens. EDB was not detected in liver, kidney, or skin on day 0 of withdrawal. EDB was not detected in whole body or tissues on day 21 of withdrawal.

IV. Distribution of EDB Residues

The total amount of EDB that was deposited into egg during days 1 to 21 of RB by each of the hens used for whole body residue analysis on day 0 of withdrawal is presented in Table 16. Two of the hens deposited only small amounts of EDB into egg because they went out of production. The 2 hens which remained in production during residue build-up deposited per hen a total of 15.5 ug of EDB into egg and 22.3 ug of EDB into whole body (Table 17). Thus, using data for laying hens only, a total of 37.8 ug of EDB was deposited into tissues and egg by each hen with tissues receiving 59.0% and egg 41.0% of the total burden. The amount of EDB deposited into tissues and eggs by laying hens accounts for only 0.26% of EDB intake. Since actual EDB intake was probably less than calculated intake (for reasons outlined in Section II of Results), percent of EDB intake deposited into tissues and egg is probably higher than 0.26%. However, even if EDB intake was 75% less than reported, deposition into tissues and eggs would still account for only 1% of intake. EDB not deposited was either: 1) not absorbed or 2) efficiently metabolized.

EDB which was deposited into whole body was distributed in

breast muscle and abdominal fat. The percent of total body burden found in each of these tissues is calculated as follows. The average body weight of EDB hens and the average concentration of EDB in whole body on day 0 of withdrawal were 1980 g and 10.8 ppb, respectively. The product of those 2 numbers (21.4 ug) is the average total body burden of EDB in each EDB hen at day 0 of withdrawal. Knowing that abdominal fat and breast muscle contained 54 and 0.44 ppb EDB, respectively, and assuming that hens: 1) deposited comparable amounts of EDB into all muscle and fat and 2) contained 19% fat and 50% muscle (Maynard et al. 1979), it follows that fat and muscle contained approximately 95% (20.3 ug) and 2% (0.4 ug), respectively, of the whole body residues. The 3% of residues which are unaccounted for were probably deposited in the yolks of developing follicles.

All EDB residues were withdrawn from muscle and fat by day 21 of withdrawal, i.e. 21.4 ug of EDB was mobilized from tissues during withdrawal. Using the calculation method outlined in Table 16, it can be shown that on average, a total of 2.5 ug of EDB (12% of the total body burden) was deposited into egg per hen during withdrawal. Therefore, mobilization from tissue into egg was not the primary withdrawal route. This indicates that the main route for withdrawal of EDB residues from fat and muscle must have involved mobilization from tissue followed by metabolism.

Table 18. Activity of aryl hydrocarbon hydroxylase (AHH) and aminopyrine N-demethylase (AND) in liver of broilers fed diet at 80 ppm polybrominated biphenyls (PBBs) for 7 days.

	Treatment	AHH activity[1]	AND activity[2]
Control broilers	None	103.8	0.51
		95.9	1.33
Mean ± SE[3]		$99.9 \pm 2.95_a$[5]	$0.92 \pm 0.41_a$
PBB broilers	80 ppm PBBs	745.8	2.57
		458.6	1.69
Mean ± SE[3]		$602.2 \pm 143.6_b$ (6.0)[4]	$2.13 \pm 0.44_b$ (2.3)

[1] pmoles hydroxylated benzopyrene produced/mg protein/minute.

[2] nmoles CH_2O produced/mg protein/minute.

[3] SE = standard error of the mean.

[4] The number in parentheses represents the increase in activity over controls.

[5] Numbers in the same column with a different subscript are significantly different ($P < .10$).

Table 19. Activity of aryl hydrocarbon hydroxylase (AHH) and aminopyrine N-demethylase (AND) in liver of hens on day 0 and 21 of withdrawal.

	AHH activity[1]		AND activity[2]	
	Day 0	Day 21	Day 0	Day 21
Control hens	1.02	0.63	35	142
	0.94	1.38	48	120
Mean ± SE[3]	0.98 ± 0.04$_a$[4]	1.01 ± 0.38$_a$	41.5 ± 6.5$_a$	131.0 ± 11.0$_b$
EDB hens	1.41	0.83	65	62
	1.47	0.67	80	107
	0.61	0.22	57	23
	0.79	2.12	23	231
Mean ± SE	1.07 ± 0.22$_a$	0.96 ± 0.41$_a$	56.3 ± 12.1$_a$	105.8 ± 45.1$_a$

[1] pmoles hydroxylated benzopyrene produced/mg protein/minute.

[2] nmoles CH_2O produced/mg protein/minute.

[3] SE = standard error of the mean.

[4] Means in the same row for the same enzyme with different subscripts are significantly different ($P < .01$).

V. Activity of Hepatic Mixed Function Oxidases

To confirm the validity of the assay by which activity of AHH and AND was measured, broilers at 6 weeks of age were fed diet containing a mixture of polybrominated biphenyl isomers (PBBs) that are known to induce activity of AHH and AND in poultry (Bursian et al. 1983 and Bursian and Polin 1986, personal communication). The mixture consisted of 62.8% hexabromobiphenyl, 13.8% heptabromobiphenyl, 10.8% pentabromobiphenyl, 2.0% tetrabromobiphenyl, and 11.4% of other bromobiphenyls. Two broilers were fed non-contaminated broiler starter and two were fed broiler starter at 80 ppm PBBs for 7 days. Then, broilers were killed, livers were excised, and mixed function oxidase (MFO) activity was determined. The activity of AHH and AND in livers obtained from broilers fed PBBs was, respectively, 6.0 and 2.3 times greater than that of controls (Table 18). Using the student's t-test, these increases were found to be significant (P .10). Thus, because an increase in activity was detected as was expected, it was decided that results obtained with the assay would be valid.

Activity of hepatic AHH and AND was determined in 2 control and 4 EDB hens on both day 0 and 21 of withdrawal (Table 19). It is not valid to statistically compare control and EDB MFO activity because hens were housed in different environments during the experimental period. However, residues of EDB were shown to have been withdrawn from all tissues by day 21 of withdrawal. That

implied that activity of hepatic AHH and AND in EDB hens on day 21 of withdrawal could be used as the control for activity in EDB hens on day 0 of withdrawal. This same comparison (day 21 versus day 0 of withdrawal) is also valid for control hens. The student's t-test was used to make the comparisons. AHH activity in control liver significantly increased from day 0 to 21 of withdrawal ($P < .01$). The reason for an increase in AHH activity in controls is not known. EDB hens also exhibited an increase in AHH activity from day 0 to 21 of withdrawal, but it was not found to be significant ($P > .15$) because of the high variability in response AND activity did not significantly change ($P > .25$) from day 0 to 21 of withdrawal in either control or EDB hens. Since there was no significant change in hepatic MFO activity in EDB hens from day 0 to 21 of withdrawal, it appears that activity of hepatic AHH and AND was not induced in hens which had consumed EDB-contaminated diet for 21 days.

DISCUSSION

I. Residues Versus Tolerance Limits

In review, the EPA has set the following tolerance limits for EDB: 1) 900 pbb in non-processed grains, 2) 150 ppb in processed grains and in grain-containing food products that will be cooked such as flour and cake mixes, and 3) 30 ppb in ready-to-eat grain-containing food products such as cereals and breads. At this time no tolerance limits have been established for EDB in products obtained from food-producing animals which have consumed EDB-contaminated grain. However, the possibility exists that the tolerance limits of the preceding could be expanded to apply to all food products. Therefore, it is useful to compare residues detected in eggs, muscle, and fat to the grain tolerance limits.

When eggs are considered as a ready-to-eat food, eggs obtained from hens fed diet containing 6.7 ppm EDB for 21 days would contain levels of EDB equivalent to the 30 ppb tolerance level. If eggs are combined with other products as in egg-nog, the EDB would be diluted, and residues would fall below the ready-to-eat tolerance limit. If eggs are cooked, the residues would be much less than the comparable tolerance limit.

Breast muscle does not contain per unit weight as much EDB residue as eggs or fat when hens are fed diet originating at 6.7

ppm EDB for 21 days. Muscle residues were well below all tolerance levels.

Concentration of EDB in chicken fat was greater than the tolerance limit for ready-to-eat foods and was the highest of all tissues analyzed. Fat can represent a source of EDB-contamination to humans by its use in: 1) soaps and paints, 2) poultry diets, and 3) human foods. Most uses require rendering and dilution with other goods, processes which should decrease residues below the lowest tolerance limit.

In summary, if tolerance limits would be expanded to include all food commodities one can expect that food products obtained from hens fed diet containing 6.7 ppm EDB for 21 days to contain residues below or near the lowest tolerance limit of 30 ppb, i.e. the foods would be marketable. If a linear relationship between concentration of EDB in diet immediately after mixing and tissue residues can be assumed, then the concentration of EDB in diet that would be required to increase tissue residues above the ready-to-eat tolerance limit are 6.7, 3.7, and 457 ppm for eggs, fat, and muscle, respectively. The dietary concentrations required to increaseresidues above the foods that will be cooked tolerance limit are 36, 19, and 2284 ppm EDB for eggs, fat, and muscle, respectively. Those levels of EDB are not typically found in grain products or diets. However, if chickens did consume EDB-contaminated grain to an extent such that residues increased above tolerances, a withdrawal period from the EDB source would

aid in reducing residues below tolerance limits.

II. Distribution and Metabolism

Less than one percent of EDB residues consumed were deposited into tissues and eggs. The other 99% of residues consumed were either absorbed and metabolized or were not absorbed. However, since EDB is fat soluble, it is likely that most of the residues consumed were absorbed in conjunction with fat. If that is so, since such small quantities of EDB were deposited into tissues and eggs, an efficient system for metabolizing EDB must exist in hens. If EDB is metabolized in poultry as it is in rats, glutathione and mixed function oxidases are involved in the process. In this study, it was found that the mixed function oxidases were not induced. This could be because: 1) the level of EDB exposure was low enough to be handled by normal enzyme activity or 2) mixed function oxidation is not a primary route for EDB metabolism in poultry. If glutathione (GSH) conjugation is a major route for EDB metabolism in poultry, their cancer risk is increased because the GSH-metabolite of EDB has been found to be a more potent carcinogen than EDB *per se*. It would be interesting to determine in poultry and mammals what percent of GSH-metabolites derived from EDB are excreted and what percent become involved in alkylation reactions with DNA strands.

In summary, the majority of the EDB consumed by hens was probably metabolized. This decreases human exposure to EDB because hens are not depositing large quantities of EDB into tissues and

eggs that would be consumed by humans. However, it could increase the cancer risk to the animals themselves because of formation of GSH-metabolites.

III. Research Design

If research similar to this study was to be conducted in the future, the design should involve the following. All hens should be housed in a similar environment or a pilot study should be done to determine what effect different environment has on hens so that the effect of EDB on feed consumption, body weights, egg production, egg weights, and activity of mixed function oxidases can be accurately determined. Dietary samples should be obtained during storage and feeding so that an accurate estimate of EDB intake can be determined. It would be interesting to ascertain what percent of EDB intake is absorbed, metabolized, or deposited into tissues versus what is not absorbed. Likewise, residues of EDB metabolites in tissues and eggs should be quantified, and the metabolic pathway in avian species should be outlined. Several levels of EDB could be fed for varying time periods in a factorial study so the no-effect levels for growth, production, and reproduction could be measured.

SUMMARY

The liquid fumigant EDB is: 1) strongly sorbed by grains during fumigation, 2) a carcinogen, and 3) interferes with reproductive processes. The purpose of this study was: 1) to determine if and to what extent chickens which had consumed EDB-contaminated grain would deposit EDB into tissues and eggs that could be consumed by humans and 2) to determine if a withdrawal period from the EDB source after contamination would reduce residues in tissues and eggs. Therefore, a practical-oriented study was conducted in which EDB-contaminated flour obtained from the Michigan food supply was fed to chickens in diet at 6.7 ppm EDB for 21 days followed by 21 days of non-contaminated diet (days 0 to 21 of withdrawal). Methodology was developed with a headspace GC for quantifying residues of EDB *per se* in eggs collected daily and tissues (whole body, fat, muscle, skin, liver, and kidney) obtained on day 0 and 21 of withdrawal. Detection sensitivities in all tissues and egg were at the ppb level. Less than one percent of EDB intake was deposited into tissues and eggs. Eggs contained detectable EDB by day 3 of feeding contaminated diet, reached a plateau of 28 ppb by day 8, and no longer contained detectable EDB by day 6 of withdrawal. Frying scrambled eggs reduced residues by 40.8%. Concentration of

EDB on day 0 of withdrawal in whole body, fat, and muscle was 11, 54, and 0.44 ppb, respectively. Fat contained 95% of whole body residues. EDB was not detected in liver, kidney, and skin on day 0 of withdrawal. EDB was not detected in any tissues on day 21 of withdrawal. No tolerance limits have been set for EDB in food products obtained from animals that were exposed to EDB, but if the tolerance limits for EDB in grain are applied to the tissues and eggs analyzed, residues would be below tolerances and the products could be marketed. Activity of hepatic mixed function oxidases was not induced. There was evidence that the hens efficiently metabolized EDB. This decreased the amount of EDB deposited into tissues and eggs but could have increased cancer risk to the animals through formation of glutathione metabolites that can alkylate strands of DNA.

APPENDICES

Appendix A. Codes of hens used for whole body and tissue analysis.

	Band #	Cage #	Code #[1]	Day killed[2]	Residue analysis
Control hens	24567	65	1	0	T[3],MFO[4]
	24577	66	2	21	T,MFO
	24578	67	3	0	WB[5]
	24579	68	4	21	WB
	24580	69	5	0	T,MFO
	24581	70	6	21	T,MFO
	24584	71	7	21(NL)[6]	WB
	24588	72	8	0	WB
EDB hens	24600	1	1	0(NL)	WB
	24568	2	2	0(NL)	WB
	24569	3	3	0(NL)	T,MFO
	24557	4	4	0	WB
	24559	5	5	0	WB
	24561	6	6	0	T,MFO
	24563	7	7	0(NL)	T,MFO
	24565	8	8	0	T,MFO
	24552	13	9	21(NL)	T,MFO
	24554	14	10	21	WB
	24556	15	11	21	T,MFO
	24558	16	12	21	WB
	24560	17	13	21(NL)	WB
	24562	18	14	21	T,MFO
	24567	19	15	21	WB
	24566	20	16	21	T,MFO

[1] These codes will be used to refer to hens in tables in the text or in other appendices.

[2] Represents day of withdrawal.

[3] T = tissues; tissues obtained include liver, kidney, skin, muscle, and fat.

[4] Liver used for mixed function oxidase assay.

[5] WB = whole body.

[6] Not laying for at least seven days prior to being killed.

Appendix B. Raw data for feed consumption, body weights, egg production, and egg weights.

I. Feed Consumption - grams/hen/day.

	Experimental days	Treatment	Food consumption
Control hens	0- 6	Non contaminated diet	123.4
	7-13	"	118.8
	14-20	"	124.4
	21-27[1]	"	96.5
	28-34[2]	"	112.4
	35-41[3]	"	115.5
EDB hens	0- 6	EDB-contaminated diet	112.4
	7-13	"	106.5
	14-20	"	93.9
	21-27[1]	Non-contaminated diet	95.6
	28-34[2]	"	93.5
	35-41[3]	"	94.7

[1] Days 0-6 of withdrawal.
[2] Days 7-13 of withdrawal.
[3] Days 14-20 of withdrawal.

Appendix B (con't.)

II. Body weights[1]-grams.

	Hen #	Day 0	Day 21[2]	Day 42[3]
Control hens	1	1756	1736	NA[4]
	2	2234	2222	2174
	3	1802	1712	NA
	4	1908	1942	1818
	5	1708	1788	NA
	6	2016	2106	2032
	7	2026	2142	2122
	8	2196	2222	NA
Mean ± SE[5]		1956 ± 70	1984 ± 77	2037 ± 79
EDB hens[6]	1	1816	1712	NA
	2	2015	2030	NA
	3	2098	2006	NA
	4	1965	2040	NA
	5	1890	1856	NA
	6	1646	1758	NA
	7	2225	2334	NA
	8	2015	2040	NA
	9	2168	2130	1894
	10	2080	2042	2064
	11	1680	1720	1662
	12	2130	2220	2488
	13	1800	1780	1622
	14	1685	1892	1798
	15	2025	2060	1896
	16	2010	2056	1886
Mean ± SE		1853 ± 45	1980 ± 45	1914 ± 96

[1] Values represent weights of individual hens on the specified day.

[2] Day 0 of withdrawal.

[3] Day 21 of withdrawal.

[4] NA = not alive on specified day.

[5] SE = standard error of the mean.

[6] EDB hens received EDB-contaminated diet days 0 to 21 and non-contaminated diet days 21 to 42 (0 to 21 of withdrawal).

Appendix B (con't.)

III. Weekly egg production - percent.

	Experimental days	Treatment	% production
Control hens	Acclimation	Non-contaminated diet	77.1
	0- 6	"	78.6
	7-13	"	73.2
	14-20	"	66.0
	21-27[2]	"	71.4
	28-34[3]	"	71.4
	35-41[4]	"	46.4
EDB hens	Acclimation	EDB-contaminated diet	75.0
	0- 6	"	64.3
	7-13	"	48.2
	14-20	Non-contaminated diet	38.4
	21-27[2]	"	48.2
	28-34[3]	"	53.6
	35-41[4]	"	62.5

[1] % production = [(# eggs laid per week per treatment)/(# hens per treatment X 7)] x 100.

[2] Day 0-6 of withdrawal.

[3] Days 7-13 of withdrawal.

[4] Days 14-20 of withdrawal.

Appendix B (con't.)

IV. Egg prduction by hen - percent[1].

	Hen #	Acclimation	Days 14 to 21	Days 35 to 41[2]
Control hens	1	83.3	71.4	NA[3]
	2	66.7	57.1	57.1
	3	83.3	71.4	NA
	4	66.7	57.1	85.7
	5	66.7	57.1	NA
	6	100.0	85.7	42.9
	7	66.7	57.1	0.0
	8	83.3	71.4	NA
Mean ± SE[4]		77.1 ± 4.4	66.0 ± 3.8	46.4 ± 197.9
EDB hens[5]	1	66.7	0(M[6])	NA
	2	66.7	0(M)	NA
	3	66.7	0(M)	NA
	4	83.3	57.1	NA
	5	83.3	57.1	NA
	6	83.3	28.6	NA
	7	50.0	0(M)	NA
	8	83.3	57.1	NA
	9	83.3	28.6	0(M)
	10	66.7	28.6	100.0
	11	83.3	71.4	85.7
	12	83.3	71.4	71.4
	13	66.7	0(M)	0(M)
	14	83.3	71.4	85.7
	15	66.7	71.4	71.4
	16	83.3	71.4	85.7
Mean ± SE		75.0 ± 2.6	38.4 ± 7.7	62.5 ± 14.0

[1] Values represent production of individual hens during the specified time. Percent production = [(# eggs laid per 7 days per hen)/7] X 100.

[2] Days 14 to 20 of withdrawal.

[3] NA = not alive during specified time.

[4] SE = standard error of the mean.

[5] EDB hens received EDB-contaminated diet days 0 to 21 and non-contaminated diet days 21 to 42 (0 to 21 of withdrawal).

[6] M = molting during specified time period.

Appendix B (con't.)

V. Egg weights[1] - grams

	Hen #	Acclimation	Days 14 to 20	Days 35 to 41[2]
Control hens	1	53.1	52.1	NA[3]
	2	66.4	65.9	62.5
	3	59.9	58.3	NA
	4	63.6	64.0	64.0
	5	62.3	60.3	NA
	6	61.2	61.7	56.5
	7	57.7	58.1	NE[4]
	8	62.6	61.7	NA
Mean ± SE[5]		60.9 ± 1.4	60.3 ± 1.5	61.0 ± 2.3
EDB hens[6]	1	60.0	NE	NA
	2	60.0	NE	NA
	3	57.6	NE	NA
	4	55.4	57.0	NA
	5	61.4	59.7	NA
	6	63.7	56.9	NA
	7	63.8	NE	NA
	8	58.0	56.6	NA
	9	61.7	59.8	NE
	10	65.3	62.9	67.5
	11	58.8	60.8	60.5
	12	59.0	59.8	61.8
	13	53.0	NE	NE
	14	57.9	60.1	60.4
	15	57.4	56.3	58.3
	16	64.6	61.7	64.2
Mean ± SE		59.9 ± 0.9	59.2 ± 0.7	62.1 ± 1.3

[1] Values are mean weight of eggs produced by one hen during specified time period.

[2] Days 14 to 20 of withdrawal.

[3] NA = not alive during specified time.

[4] NE = no eggs laid by a hen during specified time.

[5] SE = standard error of the mean.

[6] EDB hens received EDB-contaminated diet days 0 to 21 and non-contaminated diet days 21 to 42 (0 to 21 of withdrawal).

Appendix C. Statistical methology for linear regression[1].

A. Linear Model

$Y = Bo + B_1 + E$ where Bo = Y-intercept, B_1 = slope, E = random error.
Predicted $Y = \hat{Y} = bo + b_1x$ where bo and b_1 are estimates of Bo and B_1, respectively.
Predicted $X = \hat{X} = (Y-bo)/b_1$

B. Formulas for SS, r, bo, and b_1

X = dose = i to t where t = # of doses used to develop the dose-response line
Y = response
r_i = number of responses at each dose
n = total number of numbers = Σr_i
$SP_{xy} = \Sigma xy - (\Sigma x \Sigma y)/n$
$SS_y = \Sigma Y^2 - (\Sigma Y)^2/n$
$SS_x = \Sigma X^2 - (\Sigma x)^2/n$
$SS_R = b_1 SP_{xy}$
$SS_E = SS_y - SS_R$
SS_p = SS pure error = ΣSS_{y_i}

SS_{NL} = SS error due to non-linearity = $SS_E - SS_p$
$b_1 = SP_{xy}/SS_x$
$bo = Y - B_1X$
r_{xy} = product-moment correlation = $SP_{xy}/\sqrt{SS_xSS_y}$
$r_{xy}^* = r_{xy}[1+(1-r_{xy}^2)/2(n-4)]$ when $4<n<15$

$S^2_{y/x} = SS_E/n-2$
$S_{y/x} = (S^2_{y/X})^{\frac{1}{2}}$

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[1] All formulas are taken from Volume I of the **Design and Analysis of Experiments** (Gill 1978).

Appendix C (con't.)

C. Confidence Intervals

1) bo

$$bo \pm t_{\alpha/2,n-2}(S_{y/x})[1/n + (X-\bar{X})^2/SS_X]^{1/2}$$

2) b_1

$$b_1 \pm t_{\alpha/2,n-2}(S_{y/x})/\sqrt{SS_X}$$

3) $\hat{U}y/x$ (predicted mean response at one dose)

$$\bar{Y} + b_1(x-\bar{x}) \pm t_{\alpha/2,n-2}(S_{y/x})[1/n + (x-\bar{x})^2/SS_X]^{1/2}$$

4) $\hat{Y}/X$ (single predicted outcome)

$$\bar{Y} + b_1 (x-\bar{x}) \pm t_{\alpha/2,n-2}(S_{y/x})[1 + 1/n + (x-\bar{x})^2/SS_X]^{1/2}$$

5) $\hat{X}$ from a single unknown sample

$$\bar{X} + [b_1 (Y_0-\bar{Y})/g] \pm t_{\alpha/2,n-2}(S_{y/x})(\sqrt{h}/g)$$

where $g = b_1^2 - t^2_{\alpha/2,n-2}(S^2_{y/x})/SS_X$

$h = [(Y_0-\bar{Y})^2/SS_X] + (n+1)g/n$

Y_0 = observed response used to predict X

6) $\hat{\bar{X}}$ from >1 unknown sample

$$\bar{X} + [b_1(\bar{Y}_0-\bar{Y})/g] \pm t_{\alpha/2,n+m-3}(S'_{y/x})(\sqrt{h}/g)$$

where $g = b_1^2 - t^2_{\alpha/2,n+m-3}(S'^2_{y/x})/SS_X$

$h = [(\bar{y}_0-\bar{Y})^2/SS_X] + (n+m)g/nm$

Y_0 = one response

$\bar{Y}_0$ = mean of all responses used to predict X

m = # of responses used to calcualte $\bar{Y}_0$

$S'_{y/x} = \{[(n-2)(S^2_{y/x}) + (Y_{0_i}-\bar{Y}_0)^2]\}/n+m-3$

Appendix C (con't.)

7) r_{xy}

$Z_{xy} = (\frac{1}{2})\log_e[(1+r_{xy})/(1-r_{xy})]$ Use r^*_{xy} here if $4<n<15$
Use $Z^*_{xy} = Z_{xy} - [(3Z_{xy} + r_{xy})/4n]$ if $4<n<15$

$Z_u = Z_{upper} = Z_{xy} + (Z_{1-\alpha/2})\sqrt{1/(n-3)}$ (Z critical values are from the standard normal table)

$Z_L = Z_{lower} = Z_{xy} - (Z_{1-\alpha/2})\sqrt{1/(n-3)}$

$r_u = r_{upper} = (e^{2Z_u}-1)/(e^{2Z_u} + 1)$

$r_L = r_{lower} = (e^{2Z_L}-1)/(e^{2Z_L} + 1)$

D. Hypothesis testing with a linear regression table.

Source	df	SS	MS	f ratio	Critical value
Total	n-1	SS_y	---	---	---
Regression	1	SS_R	MS_R	MS_R/MS_E	$F_{\alpha,1,n-2}$
Error	n-2	SS_E	MS_E	---	---
-NL	$(n-2)-(r_i-1)$	SS_{NL}	MS_{NL}	MS_{NL}/MS_p	F_{α,V_{NL},V_p}
-P	(r_i-1)	SS_p	MS_p	---	---

Appendix D. Raw Data for Calibration Curves and Predicted Concentration of EDB in Unknowns for Egg, Tissues, and Diet.

I. Egg

A. Raw Data

ppb EDB (x)	Integrated area of the EDB peak (y)
0.5	0, 0, 0
1.0	0, 0, 0
1.5	2679, 0, 0
2.0	5116, 1273, 0
2.5	2571, 2991, 0
3.0	1808, 1613, 0
3.5	2368, 2057, 2727
4.0	3481, 3685, 2809
4.5	4382, 3292, 3462
5.0	3108, 3289, 4222
5.5	3182, 4634, 4067
6.0	3711, 5933, 3795
6.5	5670, 5158, 3923
7.0	4458, 4995, 5145
7.5	6453, 6573, 6488, 6123, 6939
15.0	9375, 14045, 10050, 7868, 8610
22.5	14903, 12981, 15571
30.0	20335, 22002, 22114, 13812, 15142
45.0	31445, 33981, 32972, 38512, 21867
60.0	41075, 43018, 43715, 33848, 30311
75.0	52661, 55015, 55006, 42506, 42714
90.0	62770, 69755, 68082
105.0	73322, 77724, 76094
120.0	83101, 88260, 87559

B. Prediction equation for doses from 4.5-120 ppb EDB

$\hat{y} = 707X-509 \quad r = .990 \quad r_u = .994 \quad r_L = .983$

C. Linear Regression Table for doses from 4.5-120 ppb EDB

Source	df	SS	MS	f	Critical Value
Total	59	$4.0018444X10^{10}$	--	--	--
Regression	1	$3.9192518X10^{10}$	$3.9192518X10^{10}$	2752[1]	f.001,1,58=12.06
Error	58	825,926,000	14,240,103	--	--
-NL	14	218,865,160	15,633,226	1.133[2]	f.25,14,44=1.298
-p	44	607,060,840	13,796,837		

[1]Significant at $P < .001$

[2]Not significant ($P > .25$), i.e., Non-linearity is not significant

D. Predicted Concentration of EDB in Eggs Obtained from EDB hens days 1 to 14 of residue buildup and days 1 to 21 of withdrawal[1].

Treatment	Day	Area[2]	EDB-ppb[3]
Residue Buildup	1	ND[4]	---
	2	ND	---
	3	6389	9.75
	4	9046	13.51
	5	11263	16.64
	6	14313	20.95
	7	15029	21.97
	8	18174	26.41
	9	17464	25.41
	10	16268	23.72
	11	16884	24.59
	12	19922	28.88
	13	17385	25.30
	14	17380	25.29
Withdrawal	1	11832	17.45
	2	11299	16.69
	3	7934	11.94
	4	4865	7.60
	5	5142	7.99
	6	ND	---
	7	ND	---
	8	ND	---
	9	ND	---
	10	ND	---
	11	ND	---
	12	ND	---
	13	ND	---
	14	ND	---
	15	ND	---
	16	ND	---
	17	ND	---
	18	ND	---
	19	ND	---
	20	ND	---
	21	ND	---

[1]No EDB was detected in eggs obtained from control hens

[2]Represents integrated area of the EDB peak. A single analysis was done on egg samples obtained for each day.

[3]Inversely predicted from $\hat{y}$ = 707X-509

[4]Not detected; detection limit = 4.5 ppb EDB

Appendix D (con't.)

E. Predicted Concentration of EDB in Eggs obtained from EDB hens days 15 to 21 of residue buildup.[1]

Treatment	Hen #	Area[2]	EDB-ppb[3]
Residue Buildup	1	NE[4]	---
	2	NE	---
	3	NE	---
	4	17400	25.32
	5	16294	23.75
	6	15790	23.04
	7	NE	---
	8	22645	32.73
	9	19020	27.61
	10	15714	22.93
	11	16423	23.94
	12	24287	35.05
	13	NE	---
	14	25329	36.53
	15	17527	25.50
	16	20334	29.46

[1]No EDB was detected in eggs obtained from control hens.

[2]Represents integrated area of the EDB peak. A single analysis was done on the homogenized egg sample obtained from eggs laid by each hen over days 15 to 21.

[3]Inversely predicted from $\hat{y}$ = 707X-509.

[4]No eggs laid by that hen during days 15 to 21.

F. Predicted concentration of EDB after scrambling in Eggs obtained from EDB hens days 15 to 21 of residue buildup.

Hen #	Area before scrambling[1]	EDB-ppb[2]	Area after scrambling[1]	EDB-ppb[3]
9	19020	27.61	11696	17.3
10	15714	22.93	9348	13.9
11	16423	23.94	9206	13.7
12	24287	35.05	13414	19.7

[1]Represents integrated area of the EDB peak.

[2]Concentration before scrambling. Inversely predicted from $\hat{y}$ = 707X-509.

[3]Concentration after scrambling. Inversely predicted from $\hat{y}$ = 707X-509.

II. Whole body

A. Raw data

ppb EDB (x)	Integrated area of the EDB peak(Y)
7.5	6962,4488, 3623
10	8417,9995,8826
12.5	11274,11013,9176,10305
15	16738,15555,15234

B. Prediction equation

$\hat{Y} = 1336X - 5039$ $r = .95$ $r_u = .98$ $r_L = .79$

C. Linear regression table.

Source	df	SS	MS	f	Critical value
Total	12	191038175	--	--	----
Regression	1	169998304	169998304	88.9[1]	f.001,1,11=19.7
Error	11	21039871	1912716	--	----
-NL	2	9797983	4898002	3.92[2]	f.05,2,9=4.26
-P	9	11241888	1249098	--	----

[1] Significant at $P < 0.01$.

[2] Not significant ($P > .05$)

D. Predicted concentration of EDB in whole body of EDB hens on days 0 and 21 of withdrawal. EDB was not detected in whole body of control hens.

Day of withdrawal	Hen #	Areas[1]	Average area[2]	EDB-ppb[3]
0	1	6070,7494,12571	8712	10.3
	2	9368,7498,7962,7974	8201	9.9
	5	9911,12721,9374,7927,7095	9406	10.8
	4	14150,10738,10437,8978	11076	12.1
21	10	ND[4]	--	--
	12	ND	--	--
	13	ND	--	--
	15	ND	--	--

[1] Represents integrated area of EDB peak. Multiple analyses were done on each liver.

[2] Represents average of all areas obtained for each liver.

[3] Inversely predicted from $\hat{Y} = 1336X-5039$.

[4] ND = not detected; Detection limit = 7.2 ppb EDB.

III. Liver

A. Raw data

ppb EDB (x)	Integrated area of the EDB peak (Y)
2.5	21753,17273,21892
5.0	28040,29996
7.5	35734,38319,38539
10.0	42846,46817

B. Prediction equation

$\hat{Y} = 3307X + 12279$ $r = .99$ $r_u = .995$ $r_L = .908$

C. Linear regression table

Source	df	SS	MS	f	Critical value
Total	9	877,418,893	---	---	----
Regression	1	847,549,377	847,549,377	227.0[1]	f.001,1,8=25.4
Error	8	29,869,516	3,733,690	---	----
-NL	2	1,390,616	695,308	0.15[2]	f.5,2,6=.78
-P	6	28,478,900	4,746,483	---	----

[1] Significant at $P < 0.001$.

[2] Not significant ($P > .5$).

D. Predicted concentration of EDB in livers obtained from EDB hens on days 0 and 21 of withdrawal. EDB was not detected in livers of control hens.

Day of withdrawal	Hen #	Area	EDB-ppb
0	3	ND[1]	--
	6	ND	--
	7	ND	--
	8	ND	--
21	9	ND	--
	11	ND	--
	14	ND	--
	16	ND	--

[1] Not detected; Detection limit = 0.8 ppb EDB.

IV. Kidney

A. Raw data

ppb EDB (X)	Integrated area of the EDB peak (Y)
5	14374
7.5	20033,20854
10	26204,36160
12.5	38182
15	45120,52837

B. Prediction equation

$\hat{Y} = 3388X - 2468$ $r = .96$ $r_u = .99$ $r_L = .72$

C. Linear regression table

Source	df	SS	MS	f	Critical value
Total	7	1,174,026,628	----	--	----
Regression	1	1,067,133,182	1,067,133,182	59.9[1]	f.001,1,6=35.5
Error	6	106,893,446	17,815,574	--	----
-NL	3	4,293,413	1,431,138	.042[2]	f.5,3,3=1.00
-P	3	102,600,033	34,200,011	--	----

[1] Significant ($P < .001$).

[2] Not significant ($P > .5$).

D. Predicted concentration of EDB in kidneys obtained from EDB hens on days 0 and 21 of withdrawal. EDB was not detected in kidneys of control hens.

Day of withdrawal	Hen #	Area	EDB-ppb
0	3	ND[1]	--
	6	ND	--
	7	ND	--
	8	ND	--
21	9	ND	--
	11	ND	--
	14	ND	--
	16	ND	--

[1] Not detected; Detection limit = 5.0 ppb EDB.

V. Skin

A. Raw data

ppb EDB (X)	Integrated area of the EDB peak (Y)
15	22614,20726
20	26129,29722
25	32900,37197

B. Prediction equation

$\hat{Y} = 1338X + 1458$ $r = .98$ $r_u = .99$ $r_L = .63$

C. Linear regression table

Source	df	SS	MS	f	Critical value
Total	5	196,704,315	----	--	----
Regression	1	178,984,262	178,984,262	40.4	f.005,1,4=313
Error	4	17,720,053	4,430,013	--	----
-NL	1	250,851	250,851	.043	f.5,1,3=.58
-P	3	17,469,202	5,823,067	--	----

[1] Significant ($P < .001$).
[2] Not significant ($P > .5$).

D. Predicted concentration of EDB in skin obtained from EDB hens on day 0 and 21 of withdrawal. EDB was not detected in control skin.

Day of withdrawal	Hen #	Area	EDB-ppb
0	3	ND[1]	--
	6	ND	--
	7	ND	--
	8	ND	--
21	9	ND	--
	11	ND	--
	14	ND	--
	16	ND	--

[1] Not detected; Detection limit = 15.0 ppb EDB.

Appendix D (con't.)

VI. Fat

A. Raw data

Treatment	Day[1]	Hen #	ppb EDB spike (X)	Integrated area of the EDB peak (Y)
EDB-contaminated diet	0	6	0	12763
			25	25459
			50	38188
			100	593437
		7	0	104505,108136
			100	212294,205424
			200	265485,301021
			400	531618,484062
		8	0	60130,47414
			100	78660,93797
			200	193408,202109
			400	208954,341515
		3	0	7621,12047
			25	16439,18626
			50	28191,23417
			100	45019,47725

[1] Represents the experimental day on which the hens were killed and the fat samples were taken, i.e. it is day 0 of withdrawal.

B. Standard addition prediction equations and concentration of EDB in fat samples of EDB hens.

Hen #	Prediction equation	r	$\lvert -bo/b_1 \rvert$[1]
6	$\hat{Y} = 465X + 13610$	.99	29 ppb
7	$\hat{Y} = 995X + 102,419$	.99	103 ppb
8	$\hat{Y} = 697X + 42,369$	.98	61 ppb
3	$\hat{Y} = 368X + 8800$	.99	24 ppb

[1] Represents |X-intercept| which is the predicted concentration of EDB in each fat sample on day 0 of withdrawal. EDB was not detected in control fat (detection limit = an area of 1705) on day 0 or 21 of withdrawal or in EDB fat on day 21 of withdrawal.

Appendix D (con't.)

C. Linear regression tables for each fat sample's standard additon equation.

Hen #6

Source	df	SS	MS	f	Critical value
Total	3	1,183,953,841	----	---	----
Regression	1	1,180,841,796	1,180,841,796	759[1]	f.005,1,2=198
Error	2	3,112,045	1,556,023	---	----
-NL	0	0	----	---	----
-P	2	3,112,045	----	---	----

[1] Significant (P < 0.05).

Hen #7

Source	df	SS	MS	f	Critical value
Total	7	$1.760X\ 10^{11}$	----	---	----
Regression	1	$1.733X\ 10^{11}$	$1.773\ X\ 10^{11}$	379[1]	f.001m,1m6=35.5
Error	6	$2.746X\ 10^{9}$	457,666,667	---	----
-NL	2	953,916,254	476,809,627	1.06[2]	f.25,2,4=2.00
-P	4	1,792,380,746	448,077,187	---	----

[1] Significant (P < .001).
[2] Not significant (P > .25).

Hen #8

Source	df	SS	MS	f	Critical value
Total	7	$8.8337202X\ 10^{10}$	----	---	----
Regression	1	$8.5039542X\ 10^{10}$	$8.5039542X\ 10^{10}$	155[1]	f.001,1,6=35.5
Error	6	$3.29777X\ 10^{9}$	$5.4961X\ 10^{8}$	---	----
-NL	2	2,111,252,126	1,055,626,063	2.36[2]	f.1,2,4=4.32
-P	4	1,792,380,746	448,095,187	---	----

[1] Significant (P < .001).
[2] Not significant (P > .1).

Hen #3

Source	df	SS	MS	f	Critical value
Total	7	1,513,498,150			
Regression	1	1,478,592,856	1,478,592,856	254[1]	f.001,1,6=3.55
Error	6	34,905,294	5,817,549	---	----
-NL	2	7,662,316	3,831,158	0.56[2]	f.5,2,4=.83
-P	4	27,242,978	6,810,745	---	----

[1] Significant (P < .001).
[2] Not significant (P > .5).

Appendix D (con't.)

VII. Muscle

A. Raw data

Treatment	Day[1]	Hen #	ppb EDB spike (X)	Integrated area of the EDB peak (Y)
EDB-contaminated diet	0	6	0	1668,1868
			5	24768,22681
			10	43230,41139
		7	0	3665,3612
			5	21654,22868
			10	46293,46911
		8	0	3614
			5	22258,20859
			10	47997,46236
		3	0	1123,1269
			5	21905,20262
			10	43033,42842

[1] Represents the experimental day on which hens were killed and muscle samples taken, i.e., it is day 0 of withdrawal.

B. Standard addition prediction equation and concentration of EDB in muscle samples of EDB hens.

Hen #	Prediction equation	r	$\lvert -bo/b_1 \rvert$[1]
6	$\hat{Y} = 4042X + 2351$	.99	582 ppt[2]
7	$\hat{Y} = 4296X + 2685$	.99	625 ppt
8	$\hat{Y} = 4459X + 1439$	.99	323 ppt
3	$\hat{Y} = 4174X + 868$	.99	208 ppt

[1] Represents |X-intercept| which is the predicted concentration of EDB in each muscle sample on day 0 of withdrawal. EDB was not detected in muscle on day 21 of withdrawal or in control muscle on day 0 or 21 of withdrawal (detection limit = an area of 696).

[2] Parts per trillion.

Appendix D (con't.)

C. Linear regression tables for each muscle sample's standard addition equation.

Hen #6

Source	df	SS	MS	f	Critical value
Total	4	1,644,952,568	----	---	----
Regression	1	1,633,493,472	1,633,493,472	579[1]	f.001,1,3=167
Error	3	8,459,096	2,819,699	---	----
-NL	1	4,075,171	4,675,171	2.13[2]	f.25,1,2=2.57
-P	2	4,383,925	2,191,963	---	----

[1] Significnat (P < .001).
[2] Not significant (P > .25).

Hen #7

Source	df	SS	MS	f	Critical value
Total	5	1,857,692,011	----	---	----
Regression	1	1,845,862,332	1,845,862,332	624[1]	f.001,1,4=74.1
Error	4	11,829,679	3,975,420	---	----
-NL	1	10,900,414	10,900,414	7.46[2]	f.05,1,3=10.31
-P	3	4,383,925	1,461,308	---	----

[1] Significant (P < .001).
[2] Not significant (P > .05).

Hen #8

Source	df	SS	MS	f	Critical value
Total	5	1,410,887,287	----	---	----
Regression	1	1,391,796,588	1,391,796,588	292[1]	f.001,1,4=741
Error	4	19,090,699	4,772,675	---	----
-NL	1	16,561,539	16,561,539	19.64[2]	f.01,1,3=34.12
-P	3	2,529,160	843,053	---	----

[1] Significant (P < .001).
[2] Not significant (P > .01).

Hen #3

Source	df	SS	MS	f	Critical value
Total	5	1,745,020,486	----	--	----
Regression	1	1,742,352,822	1,742,352,822	2613[1]	f.001,1,4=74.1
Error	4	2,667,664	666,916	--	----
-NL	1	1,289,040	1,289,040	2.81[2]	f.1,1,3=5.54
-P	3	1,378,624	459,541	--	----

[1] Significant (P < .001).
[2] Not significant (P > .1).

Appendix D (con't.)

VIII. Flour

A. Raw data

ppm EDB (X)	Integrated area of the EDB peak (Y)
25	9186000,8859300
30	10211000,10917000
35	12157000,12445000

B. Prediction equation

$\hat{Y}$ = 327,835X + 794167 r = .99 r_u = .998 r_L = 841

C. Linear regression table

Source	df	SS	MS	f	Critical value
Total	5	$1.1104393X\ 10^{13}$	----	---	----
Regression	1	$1.0747578X\ 10^{13}$	$1.0747578X\ 10^{13}$	120.5[1]	f.001,1,4=74.1
Error	4	$3.5681527X\ 10^{11}$	$8.9203817X\ 10^{10}$	---	----
-NL	1	$1.235887X\ 10^{10}$	$1.235887X\ 10^{11}$	1.08[2]	f.25,1,3=2.02
-P	3	$3.4445640X\ 10^{11}$	$1.148188X\ 10^{11}$	---	----

[1] Significant ($P < .001$).
[2] Not significant ($P > .25$).

D. Predicted concentration of EDB in flour.

Area[1]	EDB-ppm[2]	Average concentration-ppm
$1.1273X\ 10^{7}$	31.96	31.1
$1.0660X\ 10^{7}$	30.29	

[1] Integrated area of the EDB peak.
[2] Inversely predicted from $\hat{Y}$ = 327,835X + 794,167

Appendix D (con't.)

IX. Diet

A. Raw data

ppm EDB (X)	Integrated area of the EDB peak (Y)
5	3082200,3037900
10	4999200,4833200
15	6640100

B. Prediction equation

$\hat{Y}$ = 359,894X + 1,279,471 r = .999998 r_u = .999999 r_L = 999747

C. Linear regression table

Source	df	SS	MS	f	Critical value
Total	4	$9.0864291X\ 10^{12}$	----	--	----
Regression	1	$9.0666727X\ 10^{12}$	$9.0666727X\ 10^{12}$	1377[1]	f.001,1,3=167
Error	3	$1.975640X\ 10^{10}$	6585468567	--	----
-NL	1	$4.9971607X\ 10^{9}$	$4.9971607X\ 10^{9}$	0.68[2]	f.25,1,12=2.57
-P	2	$1.4759245X\ 10^{10}$	7,379,622,500	--	----

[1] Significant (P < .001).
[2] Not significant (P > .25).

D. Predicted concentration of EDB in diet.

Area[1]	EDB-ppm[2]	Average concentration-ppm
3,801,900	7.01	6.7
3,562,300	6.34	

[1] Integrated area of the EDB peak.
[2] Inversely predicted from $\hat{Y}$ = 359,894X + 1,279,471.

Appendix E. Preparation of microsomal isolation, Biuret, and MFO reagents.

I. Microsomal isolation reagents

1) Potassium chloride (kcl) - 150 mM

Dissolve 11.2 g of kcl (Mallinckrodt)[1] in 900 ml double-distilled water (DD H_2O). Bring to one liter with DD H_2O. Store at 4°C.

2) Homogenization buffer - pH 7.4

Dissolve 2.4 g Trizma® base (Sigma, T-1503) and 15 g kcl in 800 ml DD H_2O. Adjust the pH to 7.4 with 0.1 N hydrochloric acid. Bring to one liter with DD H_2O. Store at 4°C.

3) Tris-Hcl - pH 7.4, 200 mM

Dissolve 24.22 g Trizma® hydrochloride (Sigma, T-3253) in 800 ml DD H_2O. Adjust pH to 7.4 with 2.5 N sodium hydroxide (NaOH). Bring to one liter with DD H_2O. Store at 4°C.

II. Biuret crude protein determination reagents

1) NaOH - 6%

Dissolve 60.0 g NaOH (Mallinckrodt) in 900 ml DD H_2O. Bring to one liter with DD H_2O. Store at room temperature.

2) Bovine serum albumin (BSA) standard - 5 mg BSA/ml 150 mMkcl

Dissolve 125 mg BSA (Sigma, A-9647) in 10 ml 150 mMkcl. Mix gently to avoid foaming. Bring to 25 ml with 150 mMkcl. Divide into 5 ml aliquots. Store at -25°C.

3) Biuret reagent

Heat 250 ml DD H_2O to 60°C. Add 50.0 g sodium carbonate (Mallinckrodt). Stir vigorously to dissolve the sodium carbonate. Slowly add 86.5 g sodium citrate (Mallinckrodt). Allow the solution to cool. Dissolve 8.6 g copper sulfite $\cdot 5\ H_2O$ in 50 ml DD H_2O. Combine the 2 solutions in a 500 ml volumetric flask. Bring to 500 ml with DD H_2O. Mix well. Store at room temperature.

III. MFO reagents

1) Glucose-6-phosphate(G6P)-pH 7.0, 100 mg G6P/ml DD H_2O

Dissolve 1.0 g G6P monosodium salt (Sigma, G-7879) in 5 ml DD H_2O. Adjust pH to 20 with 1 N NaOH. Bring to 10 ml with DD H_2O. Store at -25°C.

2) Magnesium chloride ($MgCl_2$)-200 mM

Dissolve 5.0 g $MgCl_2 \cdot 6H_2O$ in 100 ml DD H_2O. Bring to 125 ml with DD H_2O. Store at 4°C.

3) Glucose-6-phosphate dehydrogenase (G6PD) - 0.5 units/µl 200 mM Tris-HCl

Add 1 ml 200 mM Tris-HCl to 500 units of G6PD (Sigma, G-6378). Store at -25°C.

4) Formaldehyde - 30 mM

Bring 500 µl of reagent grade formaldehyde (Mallinckrodt, 12m) to 100 ml with DD H_2O. Take 50 ml of this solution and bring to 100 ml with DD H_2O. Store at 4°C.

5) Dimethyl sulfoxide - potassium hydroxide (DMSO-KOH)

Prepare a 1 M KOH solution by dissolving 5.6 g KOH (Mallinckrodt) in 70 ml DD H_2O. Bring to 100 ml with DD H_2O. Prepare the DMSO-KOH solution by combining 85 ml DMSO (Mallinckrodt) with 15 ml 1M KOH. Store at room temperature.

6) Benzo(α)pyrene substrate[2] (BP)-6.4 mM

In a separatory funnel, add 128 µmoles (32.29 mg) cold BP (Sigma, B-3500) and 1600 µCi (128 µmoles) of tritium-labeled BP (Amersham, TRK.66, 12.5 µCi/µmole) to 50 ml glass-distilled hexane (Mallinckrodt). Add 25 ml DMSO-KOH:DD H_2O (1:1) to the funnel. Mix, allow the layers to separate, and discard the DMSO-KOH:DD H_2O (1:1) layer. Repeat the extraction 2 more times. Transfer the hexane layer to a 50 ml glass-stoppered tube. Dry under nitrogen. After the hexane has evaporated, fill the tube with nitrogen, stopper the tube, and store at -25°C. When the substrate is to be used, add 40 ml acetonitrile (Burdick and Jackson) and mix. If the substrate is stored dissolved in acetonitrile, it must be cleaned befure use. Dry 10 ml of the BP-acetonitrile solution under nitrogen. Suspend the BP in 10 ml glass-distilled hexane. Extract 2 times with 6 ml of 2.5 N HaOH in 40% ethanol. Dry under nitrogen. Resuspend in 10 ml acetonitrile.

7) Aminopyrine substrate[3] (AP) - 230 mg AP/ml methanol

Dissolve 2.3 g AP (Aldrich, D13910-6) in 5 ml reagent grade methanol (MCB). Bring to 10 ml with methanol. Store at -25°C.

8) Nash reagent

Dissolve 75 g ammonium acetate (Mallinckrodt) in 125 mg DD H_2O. Add 2 ml acetylacetone (Mallinckrodt) and 1.5 ml glacial acetic acid (Mallinckrodt). Bring to 500 ml with DD H_2O. Store at 4°C. Discard when it becomes yellow.

9) Zinc sulfate ($ZnSO_4$) - 20%

Dissolve 100 g $ZnSO_4$ in 400 ml DD H_2O. Bring to 500 ml with DD H_2O. Store at room temperature.

10) Barium hydroxide (BaOH) - saturated

Bring 250 ml DD H_2O to a boil. Slowly add BaOH (Mallinckrodt) until it will no longer go into solution. Filter the solution while hot through Whatman #1 filter paper into a bottle. Allow the solution to cool before capping the bottle. Store at room temperature.

11) Benzo( )pyrene cocktail

In a one gallon brown bottle, combine 1800 mg universal LCS cocktail (Fisher, Scintiverse™I), 360 ml gold label ethyl alcohol (Mallinckrodt), 150 ml dimethyl sulfoxide and 15 ml 1M acetic acid. Store at room temperature.

---

[1] The companies from which chemicals were purchased for this study are listed in parentheses.

[2] 3,4-benzopyrene

[3] 4-(dimethylamine)-1,2-dihydro-1,5-dimethyl-2-phenyl-3H-pyrazol-3-one

Appendix F. MFO assay raw data.

I. Day 0 of withdrawal

A. Biuret protein determination

1) Codes

Treatment	Band #	Liver weight-g	Sample #
EDB-contaminated	24563	53.23	1
	24569	38.45	2
	24561	38.29	3
	24565	53.23	4
Non-contaminated	24576	39.25	5
	24580	36.88	6

2) Determination of liver microsomal protein content.

Calibration curve

mg protein (X)	Absorbance (Y)	Equation for calibration curve
0	.063	$\hat{Y}$ = .042X + .055 r = .99
0	.057	
3	.170	
3	.165	
5	.285	
5	.255	

Samples

Sample #	Absorbance	mg protein 100 µl microsomes[1]	mg protein/ ml microsomes	$\bar{X}$ mg protein/ ml microsomes
1	.118	1.5	15	15.5
1	.121	1.6	16	
2	.110	1.3	13	13.5
2	.116	1.4	14	
3	.101	1.1	11	12.0
3	.110	1.3	13	
4	.122	1.6	16	16.0
4	.121	1.6	16	
5	.160	2.5	25	25.5
5	.162	2.6	26	
6	.112	1.4	14	14.0
6	.113	1.4	14	

[1] Inversely predicted from the calibration curve.

Appendix F (con't.)

3) Microsomal dilutions[1]

Sample #	microsomes-ml	200 mM Tris HCl-ml	mg protein/ 200 µl microsomes
1	1.0	2.1	1.0
2	1.0	1.7	1.0
3	1.0	1.4	1.0
4	1.0	2.2	1.0
5	1.0	4.1	1.0
6	1.0	1.8	1.0

[1] 1.0 ml of microsomes was diluted with Tris.HCl to obtain 1 mg protein/200 µl microsomes.

B. Calculation of activity of aminopyrine N-demethylase.

Calibration curve

nmoles CH_2O	Absorbance	Equation for calibration curve
0	.033	$\hat{Y}$ = .001X + .034 r = .99
0	.045	
60	.111	
60	.107	
120	.193	
120	.183	
240	.370	
240	.333	

Samples

Sample #	Absorbance	nmoles CH_2O[2]	$\bar{X}$ nmoles CH_2O	Net nmoles CH_2O/ mg protein/ 30 min.[3]	Net nmoles CH_2O/ mg protein/ minute
1_s[1]	.122	67.1	64.4	42.3	1.41
1_s	.115	61.7			
1_b	.063	22.1	22.1		
1_b	.063	22.1			
2_s	.123	67.8	64.0	44.2	1.47
2_s	.111	60.8			
2_b	.060	19.8	19.8		
2_b	.060	19.8			
3_s	.084	38.1	38.1	18.3	0.61
3_s	.084	38.1			
3_b	.062	21.3	19.8		
3_b	.058	18.2			
4_s	.107	55.6	45.3	23.6	0.79
4_s	.080	35.0			
4_b	.070	27.4	21.7		
4_b	.055	15.9			
5_s	.095	46.5	48.4	30.5	1.02
5_s	.100	50.3			
5_b	.060	19.8	17.9		
5_b	.055	15.9			
6_s	.095	46.5	48.0	28.2	0.94
6_s	.099	49.5			
6_b	.062	21.3	19.8		
6_b	.058	18.2			

[1] S = sample, b = blank

[2] Inversely predicted from the calibration curve.

[3] Calculated by substracting blank values from sample values.

C. Calculation of activity of aryl hydrocarbon hydroxylase.

Sample #	CPM[2]	CPM[3]	Efficiency[4]	dpm	pmoles BP-OH[5]	$\bar{X}$ pmoles BP-OH[6]	Net pmoles BP-OH[5,6]
Total count	287289	339169	.251	1144577	---	---	--
Total count	297011	354691	.279	1064556	---	---	--
Total count	317051	372260	.267	1187457	---	---	--
1_s[1]	5225	51975	.226	23119	131	126	65
1_s	4853	51478	.225	21569	122		
1_b	2559	49195	.225	11373	64	61	
1_b	2311	49290	.227	10181	58		
2_s	5696	53069	.229	24873	141	142	80
2_s	5909	54194	.233	25361	143		
2_b	2571	50158	.230	11178	63	62	
2_b	2480	50446	.232	10690	60		
3_s	5222	51729	.225	23209	131	123	57
3_s	4607	51505	.227	20295	115		
3_b	2628	50223	.230	11426	65	66	
3_b	2682	48783	.223	12027	67		
4_s	3493	52134	.235	14864	84	91	23
4_s	4017	52133	.232	17315	98		
4_b	2621	50088	.231	11346	64	68	
4_b	2880	50192	.229	12576	71		
5_s	4087	52850	.235	17391	98	103	35
5_s	4405	52082	.230	19152	108		
5_b	2782	49663	.226	12310	70	68	
5_b	2662	49709	.227	11727	66		
6_s	5272	51355	.223	23641	134	125	48
6_s	4754	53073	.233	20403	115		
6_b	2782	49139	.224	12420	70	77	
6_b	3306	49212	.222	14892	84		

[1] S = sample; b = blank

[2] B channel cpm before ^3H toluene spike.

[3] B channel cpm after ^3H toluene spike.

[4] Efficiency = (cpm after ^3H toulene spike-cpm before ^3H toluene spike)/2.07 X 10^5 dpm.

[5] Represents pmoles hydroxylated benzo(α)pyrene produced/mg protein/minute = $\frac{\text{dpm X 3 X 64,000}}{\text{1132197 X 30}}$

[6] Calculated by subtracting blank values from sample values.

Appendix F (con't.)

II. Day 21 of withdrawal

A. Biuret protein determination

1) Codes

Treatment	Band #	Liver weight-g	Sample #
Non-contaminated	24581	51.17	1
	24577	42.55	2
EDB-contaminated	24556	38.88	3
	24566	32.73	4
	24562	41.51	5
	24552	31.37	6
Control broilers[1]	20	---	7
	21	---	8
PBB broilers[1]	22	---	9
	23	---	10

[1] Broilers were carried as positive controls. Control broilers received non-contaminated broiler starter for 7 days prior to the assay. PBB broilers received diet at 80 ppm PBBs for 7 days prior to the assay.

2) Determination of liver microsomal protein content.

Calibration curve

mg protein (X)	Absorbance (Y)	Equation for calibration curve
0	.055	$\hat{Y}$ = .050X + .058 r = .99
0	.057	
3	.212	
3	.212	
5	.301	
5	.307	

Samples

Sample #	Absorbance	mg protein/ 100 µl microsomes	mg protein/ ml microsomes	$\bar{X}$ mg protein/ ml microsomes
1	.164	2.13	21.3	21.6
1	.167	2.19	21.9	
2	.119	1.23	12.3	12.2
2	.118	1.21	12.1	
3	.091	.66	6.6	6.6
3	.091	.66	6.6	
4	.071	.26	2.6	6.3
4	.107	.99	9.9	
5	.112	1.09	10.9	10.3
5	.106	.97	9.7	
6	.096	.77	7.7	8.1
6	.100	.85	8.5	
7	.091	.66	6.6	6.0
7	.085	.54	5.4	
8	.111	1.07	10.7	10.8
8	.112	1.09	10.9	
9	.111	1.07	10.7	9.7
9	.101	.87	8.7	
10	.083	.50	5.0	5.1
10	.084	.52	5.2	

[1] Inversely predicted from the calibration curve.

[2] PBB = polybrominated biphenyl.

Appendix F (con't.)

3) Microsomal dilutions[1]

Sample #	microsomes-ml	200 mM Tris-HCl	mg protein/ 200 µl microsomes
1	2.0	6.52	1.0
2	2.0	2.88	1.0
3	2.0	.64	1.0
4	2.0	.52	1.0
5	2.0	2.12	1.0
6	2.0	1.24	1.0
7	2.0	.40	1.0
8	2.0	2.32	1.0
9	2.0	1.88	1.0
10	2.0	.04	1.0

[1] 2.0 ml of microsomes were diluted with Tris-HCl to obtain 1 mg protein/200 µl microsomes.

Appendix F (con't.)

B. Calculation of activity of aminopyrine N-demethylase.

Calibration curve

nmoles CH2O	Absorbance	Equation for calibration curve
0	.040	$\hat{Y}$ = .002X + .034 r = .99
0	.040	
60	.129	
60	.130	
120	.230	
120	.225	
240	.440	
240	.430	

Samples

Sample #	Absorbance	nmoles CH_2O[2]	$\bar{X}$ nmoles CH_2O	Net nmoles CH_2O mg protein/ 30 min.[3]	Net nmoles CH_2O mg protein/ minute
1_s[1]	.112	47.0	61.5	18.9	0.63
1_s	.160	76.0			
1_b	.065	18.6	42.6		
1_b	.074	24.0			
2_s	.122	53.0	49.1	41.3	1.38
2_s	.109	45.2			
2_b	.040	3.4	7.8		
2_b	.055	12.5			
3_s	.122	53.0	41.5	24.8	0.83
3_s	.084	30.0			
3_b	.062	16.7	16.7		
3_b	.062	16.7			
4_s	.103	41.5	42.1	20.2	0.67
4_s	.105	42.7			
4_b	.070	21.6	21.9		
4_b	.071	22.2			
5_s	.081	28.2	25.8	6.6	0.22
5_s	.073	23.4			
5_b	.070	21.6	19.2		
5_b	.062	16.7			
6_s	.205	103.2	101.7	63.5	2.12
6_s	.200	100.2			
6_b	.110	45.8	38.2		
6_b	.085	30.6			
7_s	.100	39.7	39.1	15.4	0.51
7_s	.098	38.5			
7_b	.072	22.8	23.7		
7_b	.075	24.6			
8_s	.131	58.5	57.9	39.9	1.33
8_s	.129	57.2			
8_b	.072	22.8	18.0		
8_b	.056	13.1			
9_s	.174	84.4	86.6	77.1	2.57
9_s	.181	88.7			
9_b	.050	9.5	9.5		
9_b	.050	9.5			
10_s	.131	58.5	66.1	50.6	1.69
10_s	.156	73.6			
10_b	.060	15.5	15.5		
10_b	.060	15.5			

[1] S = sample; b = blank.

[2] Inversely predicted from the calibration curve.

[3] Calculated by subtracting blank values from sample values.

Appendix F (con't.)

C. Calculation of activity of aryl hydrocarbon hydroxylase.

Sample #	cpm[2]	cpm[3]	Efficiency[4]	dpm	pmoles BP-OH[5]	$\bar{X}$ pmoles BP-OH[5]	Net pmoles BP-OH[5,6]
Total count	228457	290545	.300	761523	---	---	---
Total count	280730	346417	.317	885584	---	---	---
Total count	237803	302349	.312	762189	---	---	---
1_s[1]	6046	55674	.240	25192	201	177	143
1_s	4592	53971	.239	19213	153		
1_b	1019	49610	.235	4336	35	34	
1_b	1016	49915	.236	4305	34		
2_s	4638	53908	.238	19487	155	155	120
2_s	4665	54489	.241	19357	154		
2_b	1106	50709	.240	4608	37	35	
2_b	993	50637	.240	4138	33		
3_s	3078	53486	.244	12615	101	95	61
3_s	2738	52839	.242	11314	90		
3_b	1065	50474	.239	4456	36	34	
3_b	967	50588	.240	4029	32		
4_s	4362	53515	.237	18405	147	139	107
4_s	3967	53558	.240	16529	132		
4_b	1028	50994	.241	4266	34	32	
4_b	906	51757	.246	3683	29		
5_s	1718	51135	.239	7188	57	58	23
5_s	1757	51399	.240	7321	58		
5_b	1115	50977	.241	4627	37	35	
5_b	985	50857	.241	4087	33		
6_s	7478	57040	.239	31289	249	264	231
6_s	8097	55939	.231	35052	279		
6_b	989	50608	.240	4121	33	33	
6_b	1000	51345	.243	4115	33		
7_s	4006	54066	.242	16554	132	136	104
7_s	4345	55384	.247	17591	140		
7_b	1029	51505	.244	4217	34	32	
7_b	940	51956	.246	3821	30		
8_s	4141	54644	.244	16971	135	131	96
8_s	3895	54212	.243	16029	128		
8_b	1073	50905	.241	4452	35	35	
8_b	1056	50405	.238	4437	35		
9_s	23299	73640	.243	95881	764	781	745
9_s	24151	74042	.241	100212	799		
9_b	1013	51922	.246	4118	33	36	
9_b	1175	51493	.243	4835	39		
10_s	15146	64315	.238	63639	507	493	459
10_s	14165	62984	.236	60021	478		
10_b	1047	51528	.244	4291	34	34	
10_b	1055	51633	.244	4324	34		

[1] S = sample; b = blank.

[2] B channel cpm before ^3H toluene spike.

[3] B channel cpm after ^3H toluene spike.

[4] Efficiency = (cpm after ^3H toluene spike - cpm before ^3H toluene spike)/2.07 X 10^5 dpm.

[5] Represents pmoles hydroxylated benzo(α)pyrene produced/mg protein/minute

$$= \frac{\text{dpm X 3 X 64,000}}{\text{803,099 X 30}}$$

[6] Calculated by subtracting blank values from sample values.

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