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AN EXPERIMENTAL MODEL OF HEPATIC FIBROSIS INDUCED BY THE ADMINISTRATION OF DIBUTYLTIN DICHLORIDE

Jeannee Karen Yermakoff
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AN EXPERIMENTAL MODEL OF HEPATIC FIBROSIS

INDUCED BY THE ADMINISTRATION OF

DIBUTYLTIN DICHLORIDE

BY

JEANNEE KAREN YERMAKOFF

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE

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MASTER OF SCIENCE THESIS

OF

JEANNEE KAREN YERMAKOFF

Approved:

Thesis Committee

Major Professor _George C Fuller_

Leonard W Foster

_____

A. A. Michel

Dean of the Graduate School

UNIVERSITY OF RHODE ISLAND

1978

ABSTRACT

Yermakoff, Jeannee Karen. M.S., University of Rhode
Island, 1978. An Experimental Model of Hepatic Fibrosis
Induced by the Administration of Dibutyltin Dichloride.
Major Professor: Dr. George C. Fuller.

Dibutyltin dichloride (DBT), one of a series of dialkyl-
tin derivatives used industrially as polyvinylchloride
stabilizers and urethane catalysts, was investigated for its
ability to induce liver fibrosis in rats.

Collagen, the predominant protein in fibrotic tissue,
is synthesized in a series of sequential steps consisting
of assembly of a proline-rich and lysine-rich polypeptide
precursor of collagen, enzymatic hydroxylation of some of
the prolyl and lysyl residues, and glycosylation of some of
the hydroxylysyl residues. Since prolyl residues are not
hydroxylated before they are in peptide-bound form, the
conversion of isotopically labeled proline to hydroxyproline
by prolyl hydroxylase was taken as one parameter reflecting
the rate of collagen formation. In vitro collagen synthesis
was determined by incubation of liver biopsies with labeled
proline, after which incorporation of label into collagenase
digestible protein is compared to incorporation into col-
lagenase-resistant protein. Hydroxyproline content served
as a parameter of increased collagen accumulation, since
hydroxyproline does not appear in significant amounts in
noncollagen protein.

DBT was administered by oral intubation (10 and 20 mg/kg) every other day for 12 days. Histopathological observation in these rats revealed extensive inflammation in portal tracts, bile duct inflammation and proliferation, fibrosis, necrosis, infarcted areas and granulomatous lesions. In the higher dose group, a greater than two-fold increase in hydroxyproline content, a greater than 50% increase in prolyl hydroxylase activity and a two-fold increase in relative collagen synthesis in vitro was observed at the end of the 12 day period.

DBT, administered by oral intubation (10 and 20 mg/kg) daily for four days, produced inflammation of portal tracts and bile duct inflammation and proliferation. Prolyl hydroxylase was increased 50 and 130% over control values in the 10 and 20 mg/kg groups, respectively. In vitro collagen synthesis increased approximately five-fold in the higher dose group. However, there was no increase in relative collagen synthesis due to a concomitant elevation in noncollagen protein, probably as a result of inflammatory cell protein synthesis.

DBT (3×10^{-7} and 3×10^{-6} M) had no effect on prolyl hydroxylase activity of L929 mouse fibroblasts in cell culture.

ACKNOWLEDGEMENTS

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INTRODUCTION

The toxicity of alkyltin derivatives has been
recognized for some time. Intensive investigation of
the biological effects of alkyltin compounds began in
the mid 1950's in response to increased industrial use
of these compounds and a disaster in France caused by
the inclusion of an alkyltin derivative in a preparation
for the treatment of skin infections. Currently, di-
alkyltin compounds are widely used as polyvinylchloride-
stabilizers in medicinally-used plastics, the biological
inertness of which has been questioned.

Dialkyltin derivatives produce hepatic and biliary
damage (Barnes and Stoner, 1958; Barnes and Magee, 1958;
Gaunt et al., 1968). Barnes and Magee (1958) reported
hepatic fibrosis in rats in addition to inflammation and
bile duct injury following the administration of dibutyl-
tin dichloride (DBT).

Hepatic fibrosis has long been recognized as one
of the sequelae of inflammation and chemically-induced
liver damage. This investigation was conducted to
determine the fibrogenic potential of DBT in rat liver
and the relationship of this fibrogenesis to associated
histopathological changes. In vivo and in vitro labora-
tory models were employed for measurement of hepatic

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collagen synthesis following the oral administration
of DBT.

LITERATURE SURVEY

Collagen Biosynthesis

Collagen, the fibrous protein component of connective tissue, is present in the extracellular matrix as rigid fibers made up of cross-linked collagen molecules. These molecules each consist of three polypeptide chains coiled into a unique left-handed triple helix. Five different types of polypeptide chains, called α chains, have been characterized in four different types of collagen (Crystal, 1974; Miller, 1976). Type I collagen, found primarily in bone and skin, consists of two $\alpha 1$ chains and one $\alpha 2$ chain, which are distinguishable by their separation on carboxymethylcellulose columns, and is represented as $[\alpha 1(I)]_2$ 2. The remaining three types of collagen each consist of three identical $\alpha 1$ chains, with each type of $\alpha 1$ chain differing slightly in amino acid composition. Type II collagen ($[\alpha 1(II)]_3$) is found primarily in cartilage; type III ($[\alpha 1(III)]_3$) in fetal tissue, muscle, and aorta; and type IV ($[\alpha 1(IV)]_3$) in basement membranes.

Collagen biosynthesis consists of the assembly of a proline- and lysine-rich polypeptide precursor of collagen, procollagen, which is subsequently modified by a number of

post-translational modifications. Hydroxylation of both
proline (Prockop and Juva, 1965) and lysine (Sinex et al.,
1959) has been shown to occur subsequent to incorporation
of these amino acids into procollagen chains. Following
hydroxylation of lysine, some of these residues are glyco-
sylated to galactosylhydroxylysine and glucosylgalactosyl-
hydroxylysine (Spiro and Spiro, 1971, 1971[a], and 1971[b]).

Cellular processing of procollagen α chains also
includes interchain disulfide-bond linkage in non-helical
regions of the molecule (Bornstein, 1974) which is essential
for triple-helix formation (Speakman, 1971). Following the
secretion of procollagen, these non-helical regions are
removed by the extracellular enzyme, procollagen peptidase
(Lapiére et al., 1971; Goldberg et al., 1975). Collagen
cross-linking occurs through additional extracellular
processing by the enzyme lysyl oxidase which oxidatively
deaminates specific lysine residues leaving aldehyde
moieties which form cross links through condensation
reactions (Siegal et al., 1970).

Hepatic Fibrosis in Liver Disease

Collagen is the most abundant protein in the human
body and the major protein of connective tissue. Normal
liver, however, contains only a small amount of connective
tissue. Hepatic fibrosis defines an excess of collagenous

fibrous tissue in the liver (Leevy et al., 1976). In-
creased collagen content in human and experimental liver
disease leading to cirrhosis has been established by
histological observation and chemical determination of
hydroxyproline (Kent et al., 1959). Excess collagen
accumulation may occur subsequent to the collapse of
preformed collagen fibers or from formation of new fibers.
However, collapse and aggregation of existing fibrous
tissue following hepatocyte necrosis are thought to play
a minor role in hepatic fibrosis compared to formation of
new fibers (Popper and Udenfriend, 1970). Increased
de novo synthesis of collagen has been associated with
the accumulation of collagen in experimental cirrhosis
(Huberman et al., 1969).

An increase in hepatic collagen may result from one
or a combination of three processes: increased collagen
biosynthesis; increased maturation (cross-linking), fibril
formation and deposition of collagen; and decreased
catabolism of fibers.

Increased collagen biosynthesis has been demonstrated
as increased activity of the intracellular enzyme prolyl
hydroxylase in experimental (Takeuchi et al., 1976) and
human cirrhosis (Fuller et al., 1976; Fuller et al., 1977).
Additional evidence for increased collagen biosynthesis
in hepatic fibrosis has been obtained by measurement of

in vitro incorporation of labeled proline into collagen
in human (Chen and Leevy, 1976) and experimental (Rodjkind
and Diaz De Leon, 1970) liver biopsies. Increased collagen
biosynthesis has also been correlated with an increase in
the free proline pool and concomitant decrease in the amount
of free glutamic acid, a precursor of proline, in both
experimental (Rodjkind and Diaz De Leon, 1970) and human
cirrhosis (Kershinobich et al., 1970). Fibroblasts were
long thought to be responsible for increased collagen bio-
synthesis in hepatic fibrosis. Recently, however, enzymes
responsible for collagen biosynthesis have been found in
many nonfibroblastic cell lines in culture (Langness and
Udenfriend, 1974). In the liver, vitamin A-containing
perisinusoidal cells (Ito cells or lipocytes) have been
implicated in fibrogenesis by serving as resting precursors
of fibroblasts (Kent et al., 1976).

Increased extracellular maturation and deposition
of collagen may occur in hepatic fibrosis due to the
increased availability of surfaces with reduced solid and
fluid exchange, which serve as anchors for collagen fibers
(Popper and Udenfriend, 1970). Structures which may
contain surfaces such as these include the sinusoidal
surface of damaged hepatocytes and the basement membranes
of proliferated bile ductules and capillaries.

The role of collagen resorption in hepatic fibrosis
remains to be established. Extracellular collagenase in

the liver has been observed by immunohistochemical
methods primarily in the liver capsule and portal tracts
(Montfort and Perez-Tamayo, 1975); intracellular colla-
genase in an active form has been reported to be present
in Kupffer cells of the hepatic reticuloendothelial
system (Fujiwara et al., 1973). Hepatic collagenase
activity has been reported to be increased in carbon
tetrachloride-induced experimental fibrosis, primarily
in the subacute stage of treatment (Okazaki and Marujama,
1974).

Necrosis and inflammation may be primary stimuli of
fibrogenesis. The hypothesis that hepatocyte death is
fibrogenic has been accepted for some time (Aterman, 1954).
Hepatic necrosis has been associated with fibrogenesis in
studies with such compounds as carbon tetrachloride
(Cameron and Karunaratne, 1936) and dimethylnitrosamine
(Madden et al., 1970). Death of hepatocytes has been
thought to cause fibrogenesis by release of either macro-
or micromolecular products from these cells (Popper, 1975).
Inflammation has been implicated as a precursor to fibro-
genesis. Pharmacologically active compounds formed by
inflammatory cells may act as stimuli of fibrogenesis
(Popper, 1975). Immune complexes have been suggested as
stimuli of fibrogenesis, since localization of such com-
plexes in portal tracts preceded the development of
cirrhosis in rats induced by immunization with heter-
ologous sera (Paronetto and Popper, 1966).

Conversely, necrotic and inflammatory processes often associated with hepatic fibrosis may be initiated by the functional changes resulting from collagen accumulation. Anoxic injury to hepatocytes occurs from fibrosis and the associated circulatory impairment; the resulting breakdown products of dead hepatocytes may in turn be responsible for stimulation of the inflammatory process.

Organotin Toxicity

Organotin derivatives have demonstrated usefulness as biocidal compounds, heat stabilizers and catalytic agents (Piver, 1973). Dialkyltin compounds are used primarily as heat stabilizers and catalysts in polyvinylchloride and polyurethane production, respectively; trialkyl- and triaryltin derivatives are employed as biocidal agents. Such applications of organotin compounds have resulted in increased industrial use whereby these compounds are now the fourth largest group of organometallics produced.

Dibutyltin derivatives have not been sanctioned by the Food and Drug Administration for use in plastics which come in contact with food; dioctyltin compounds have been developed for this purpose (Piver, 1973). However, dibutyltin derivatives are widely used as polyvinylchloride stabilizers in medicinally-used plastics.

The biological inertness of plastics used in the medical, dental and pharmaceutical professions has been questioned (Nimni, 1964; Guess and Stetson, 1968; Guess and Haberman, 1968). Organotin-containing polyvinylchloride plastics implanted both intramuscularly and subcutaneously in rats and rabbits causes a local tissue reaction manifested by necrosis, proliferation of granulation tissue and encapsulation by fibrous tissue (Nimni, 1964). Injection of extracts of these plastics and dilutions of up to 1:10,000 of dibutyltin dilaurate produced a similar inflammatory response implicating dibutyltin dilaurate as the toxic component. Guess and Haberman (1968) investigated the toxicity of plastics and their additives by cell culture techniques. All ten of the dibutyltin stabilizers studied demonstrated some degree of toxicity.

Extensive investigation of the toxicity of organotin derivatives began in the mid 1950's. The impetus for this was the increased industrial use of these compounds and the "Stalinon" disaster in France in 1954 (Barnes and Stoner, 1961). "Stalinon" was a preparation sold for the treatment of staphylococcal skin infections, which was said to contain 15 mg diethyltin diiodide and 100 mg linoleic acid per capsule, but later was found to contain large quantities of mono- and triethyltin. The

recommended dose was six capsules per day for eight days. Two hundred and seventeen poisonings resulting in 100 deaths were reported due to "Stalinon". Symptoms included persistent headache, vertigo, visual and psychic disturbances, vomiting and abdominal pain. Autopsy revealed interstitial cerebral edema and some venous endothelial proliferation with thrombosis and hemorrhages.

Investigation of a series of alkyltin derivatives revealed that, with few exceptions, the toxicity of these compounds increases as the size of the alkyl group decreases and that trialkyl derivatives exhibit greater toxicity than their dialkyl counterparts (Stoner et al., 1955).

Trialkyltin compounds have been shown to produce interstitial edema of the white matter of the brain in rats (Magee et al., 1957) and inhibit oxidative phosphorylation in vitro (Aldridge and Cremer, 1955; Aldridge and Street, 1964, 1970, and 1971). Inhibition of muscular contractility in vitro associated with disruption of mitochondria, disorganization of muscle fibers and depletion of neuromicrotubules has also been observed (Tan and Ng, 1977).

Dialkyltin differ from trialkyltin compounds in that the primary lesion of these compounds is biliary and hepatic damage (Barnes and Stoner, 1958; Barnes and Magee, 1958; Gaunt et al., 1968). Dibutyltin is the most active

of the dialkyltin series in this respect. Barnes and
Magee (1958) reported bile duct damage and liver inflam-
mation two days after oral administration of dibutyltin
dichloride (50 mg/kg for three days). After six months,
the bile duct was shortened and thickened and hepatic
portal fibrosis was evident.

EXPERIMENTAL

Animals

Male Long Evans rats weighing 250-350 grams were purchased from Charles River Breeding Laboratories, Inc. (Wilmington, MA). The rats were housed in groups of six in colony cages at an ambient temperature of 24-27° C. with alternating 12 hour light/dark cycles. Rats were fed Purina Laboratory Chow and water ad libitium.

Materials

Di-n-butyltin dichloride was purchased from Eastman Kodak Co. (Rochester, NY). The radioactive amino acids, $4-^3H$-proline (specific activity 25-30 Ci/mmole) and $2,3-^3H$-proline (specific activity 20 Ci/mmole) were purchased from New England Nuclear Corporation (Boston, MA). Bacterial collagenase, free of nonspecific proteases, was purchased from Advanced Biofactures (Lynbrook, NY). Eagles Minimum Essential Medium was obtained from Grand Island Biological Company (Grand Island, NY). Analytical grade reagents were used throughout the investigation.

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Cell Culture Techniques

An established line of L929 fibroblast cells were maintained in monolayer cultures in Eagles Minimum Essential Medium with 10% fetal calf serum and chlortetracycline (50 ug/ml). Treated cell cultures were incubated with dibutyltin dichloride (0.1 mg/ml in 25% ethanol) during late log phase at final concentrations of 3×10^{-7} M and 3×10^{-6} M. Control cultures received equivalent amounts of 25% ethanol. After 24 hours, medium was decanted and cells harvested by centrifugation following the addition of 0.1% trypsin. The pellet was washed three times with phosphate buffered saline and disrupted by ultrasonic energy (30 watts for two 15 second periods) in 1 ml 0.25 M sucrose. Prolyl hydroxylase activity was determined in 50 and 100 ul aliquots of cell sonicates.

Dibutyltin-Induced Hepatic Fibrosis

Fibrosis and associated liver injury was produced in male Long Evans rats by the administration of dibutyltin dichloride in corn oil by oral intubation. Dibutyltin dichloride was administered daily in animals treated for four days and every two days in animals treated for 12 days. Rats were sacrificed by decapitation 24 hours after the last administration of dibutyltin dichloride. The

livers were removed and quickly chilled. Sections of liver tissue were fixed and prepared for light microscopy using hematoxylin-eosin and trichrome stains. Liver samples were also prepared for determination of prolyl hydroxylase activity and in vitro collagen synthesis. Remaining liver tissue was stored frozen for determination of hydroxyproline content.

Hydroxyproline Determination

Liver hydroxyproline content was determined using the method of Kivirikko et al. (1967) with some modifications. Acid hydrolyzates of liver were prepared by autoclaving 150 mg liver in 4 mls 6 N HCl for 24 hours. Following hydrolysis, samples were decolorized with activated charcoal, centrifuged at low speed (1000 rpm) and the resultant supernates filtered. Using phenolphthalein as an indicator, samples were adjusted to a pink color with 1 and 10 N potassium hydroxide. Aliquots containing 3-15 ug hydroxyproline and hydroxyproline standards (5-25 ug) were diluted to 4 mls. The pH was again adjusted using phenolphthalein as an indicator and solutions were saturated with potassium chloride. One ml potassium borate buffer (pH 8.7) and 0.5 ml 10% alanine were added with mixing. After 30 minutes samples were oxidized with 1 ml 0.2 M chloramine-T solution at room temperature.

The reaction was stopped after 25 minutes with 3 mls 3.6 M sodium thiosulfate. Toluene-soluble oxidation products were extracted in 5 mls toluene and discarded. Pyrrole was formed from oxidation products of hydroxy-proline remaining in solution by heating to 100° C for 30 minutes. After cooling, pyrrole was extracted with 5 mls toluene. The addition of 1 ml Ehrlich's reagent (p-dimethylaminobenzaldehyde) to 2.5 mls extract produced a pyrrole chromophore after 30 minutes at room temperature with a characteristic absorbance at 560 mu.

Preparation of Substrate for Prolyl Hydroxylase Assay

The tritium labeled substrate was prepared by the method of Hutton et al. (1966) with some modifications. Twenty-five to thirty dozen, 7-day-old decapitated chick embryos were placed in ice-cold Krebs-Ringer buffer[a] (Stone and Meister, 1962). After washing twice with ice-cold Krebs, embryos were minced in the presence of a small amount of ice-cold Krebs. Five to six gram portions of minced embryo were placed in 50 ml beakers and enough Krebs buffer was added to bring the final volume to 20 mls. Following the addition of 1.0 mci

[a]NaCl, 1.285 g; KCl, 0.224 g; $MgSO_4$, 0.144; $CaCl_2$, 0.144 g; KH_2PO_4, 0.544 g; $NaHCO_3$, 2.10 g; D-Glucose, 1.80 g; and distilled water to 1 liter.

$4-^3$H-proline and 20 ul of 1.0 M α,α-dipyridyl, the mixture was incubated for two hours at 37° C under O_2-CO_2 (95/5%) in a metabolic shaker. After two hours, the embryo minces were centrifuged at 30,000 rpm for 90 minutes and the supernates discarded. Newly synthesized collagen was extracted from the pellet with 0.5 M acetic acid (2 ml/gm tissue) overnight. After centrifugation for 90 minutes at 30,000 rpm, the supernate containing newly-formed, unhydroxylated collagen was dialyzed against ten volumes of 0.01 M Tris HCl buffer (pH 7.4) with at least four changes. The substrate was then autoclaved for 15 minutes and redialyzed against three changes of 10 volumes of Tris HCl buffer (pH 7.4). Substrate was then calibrated for specific activity and linearity with a standard purified rabbit enzyme (Chichester et al., 1976) and frozen in 10 ml aliquots.

Prolyl Hydroxylase Activity

Immediately after rats were sacrificed, livers were removed and 15-25 mg aliquots of tissue were homogenized in 300 ul of buffer containing 0.25 M sucrose, 10^{-5} M ethylenediaminetetraacetic acid, 10^{-5} M dithiothreitol and 0.01% Triton. Prolyl hydroxylase activity in 50 and 100 ul aliquots of homogenate was determined by the method

of Hutton et al. (1966) with some modifications. Aliquots
of liver homogenates were incubated at 30° C for 30 min-
utes in a mixture containing approximately 600,000 cpm
(depending on substrate batch) of tritiated substrate
(0.2 ml), 0.5 mM ascorbate, 0.1 mM α-ketoglutarate,
0.1 mM ferrous ammonium sulfate, 0.05 M Tris HCl buffer
(pH 7.4), 0.2% bovine serum albumin and 0.02% catalase.
The prolyl hydroxylase reaction was stopped after 30
minutes by the addition of 0.1 volume 50% trichloroacetic
acid. Tritiated water was then collected by vacuum distilla-
tion; a measured fraction of tritiated water was counted
for radioactivity in a Packard Tri-Carb Liquid Scintillation
Spectrometer (Model 3310). This radioactivity reflects
the stoichiometric formation of hydroxyproline and tritiated
water by prolyl hydroxylase in the presence of cofactors
and labeled substrate as described above (Rhoads and
Udenfriend, 1970). Due to variability in substrate batches,
a conversion factor, derived from substrate calibration
with standard enzyme, is employed to permit comparisons of
prolyl hydroxylase activity values when different substrate
preparations are used.

In Vitro Collagen Biosynthesis

Collagen and noncollagen protein synthesis by liver
tissue in vitro was determined by the method of Diegelmann
et al. (1975) with modifications. Approximately 100 mg of
freshly dissected liver minces were incubated for 24 hours
at 37° C under O_2-CO_2 (95/5%) in 4 mls of Eagles minimum
essential medium containing 4-^{3}H- or $2,3$-^{3}H-proline (50
uci/ml), 10^{-4} M ascorbate and 50 ug/ml β -aminoproprio-
nitrile. Experiments have been conducted confirming that
label incorporation into collagen and noncollagen protein
is linear against time between 6 and 24 hours; the percent
label in collagen does not change during this 24 hours.
The incubation mixture was homogenized in a Bellco glass/
glass homogenizer and protein was precipitated by the addi-
tion of 0.1 volume 50% trichloroacetic acid at 4° C. The
pellet, collected by centrifugation at 5,000 rpm for 30
minutes, was resuspended in 0.5 M acetic acid and dialyzed
against 100 volumes of 0.5 M acetic acid with two changes
and against 100 volumes of 0.05 M Tris HCl buffer (pH 7.4)
with two changes to remove unincorporated labeled proline.
Tubes of 0.4 ml aliquots of sample were then incubated for
18 hours at 37° C in a mixture containing 0.05 M Tris HCl
buffer (pH 7.4), N-ethylmaleimide (2.5 umole), calcium
chloride (0.5 umole), 0.1 ml chloroform and 130 units of
bacterial collagenase. Duplicate tubes were incubated
without collagenase, which served as enzyme blanks.

Following incubation, the reaction was stopped and protein was coprecipitated with 0.1 volume bovine serum albumin (1.0%) by the addition of one volume 10% trichloroacetic acid. After 30 minutes on ice, samples were centrifuged at 5,000 rpm for 30 minutes. The pellet was digested in 2 mls Digestol[R] (Yorktown, Hackensack, NJ) and counted in 10 mls toluene-based scintillation cocktail. The 5% trichloro-acetic acid supernate was again coprecipitated using bovine serum albumin (1.0%) and counted in 10 mls Hydromix[R] (Yorktown, Hackensack, NJ). Protein content was determined on liver homogenates. Collagen and noncollagen protein synthesis per unit protein was calculated from collagenase-digested protein in the supernate and collagenase-resistant protein in the pellet, respectively. Relative collagen synthesis was calculated using a formula which accounted for the relative enrichment of collagen proline/hydroxy-proline content (5.4 times) compared to noncollagen protein (Diegelmann et al., 1975).

Protein Determination

Protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin as the protein standard. The intensity of the characteristic blue color was read at 750 mu on a Gilford Model 250 Spectrophotometer.

Statistical Methods

Students' t-test was used to test for significant differences between sample means. The sample means, standard errors of the means and t-statistics were calculated on an Olivetti-Underwood Programma 101 computer using the following formulae.

a.) Arithmetic means of sample:

$$\bar{X}_1 = \frac{\Sigma X_1}{N_1} \; , \; \bar{X}_2 = \frac{\Sigma X_2}{N_2}$$

b.) Estimated standard error of the mean:

$$\hat{\sigma}_{\bar{X}_1} = \sqrt{\frac{S_1}{N_1 - 1}} \; , \; \hat{\sigma}_{\bar{X}_2} = \sqrt{\frac{S_2}{N_2 - 1}}$$

c.) t-statistic:

$$t = \frac{\left| \bar{X}_1 - \bar{X}_2 \right|}{\hat{\sigma}_{\bar{X}_1 - \bar{X}_2}}$$

Acceptable level of significance in these studies was defined as $P < 0.05$ for a one-tailed t-test.

RESULTS

Cell Culture Investigation

The effect of dibutyltin dichloride (DBT) on mouse fibroblasts in cell culture is shown in Table 1. When L929 fibroblasts were incubated with DBT at concentrations of 3×10^{-7} M and 3×10^{-6} M at late log phase, no change in prolyl hydroxylase activity in cell sonicates was observed compared to control values.

Liver Hydroxyproline Content

Since hydroxyproline does not appear in significant amounts in noncollagen protein, the amount of this amino acid reflects the amount of collagen present in liver tissue. The effect of DBT on liver hydroxyproline content in rats following treatment every two days for 12 days is shown in Table II. The amount of hydroxyproline, expressed as ug/mg liver wet weight, reflects measurement of the pyrrole chromophore of this amino acid in liver acid hydrolyzates. In the 10 mg/kg-treatment group, there was no change in liver hydroxyproline content.

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TABLE I

Effect of Dibutyltin (DBT) on Prolyl Hydroxylase [a]
Activity in L - 929 Cells

Group	prolyl hydroxylase activity[b] cpm/ug protein
Control	54
	60
DBT (3 x 10^{-7}M)	56
	60
DBT (3 x 10^{-6}M)	47
	64

[a]late log phase L-929 cells were incubated with DBT for 12 hours.

[b]estimated by the formation of ^{3}HHO from incubation at 30°C of
4-^{3}H-proline rich substrate and cofactors with 50 and 100 ul
aliquots of cell sonicates prepared as described in the methods.
Each value represents mean of enzyme activity measurements from
one flask of cells reported as cpm/ug protein/30 min.

TABLE II

Effect of Dibutyltin (DBT) on Liver Hydroxyproline Content
in Rats After 12 Days of Treatment [a]

Group	N[b]	Hydroxyproline[c] ug/mg liver wet weight
Control	6	0.125 $\pm$ 0.004 (range 0.105 - 0.133)
DBT (10 mg/kg)	7	0.148 $\pm$ 0.019 (range 0.102 - 0.249)
DBT (20 mg/kg)	6	0.266 $\pm$ 0.044[d] (range 0.157 - 0.447)

[a]DBT in corn oil was administered every 2 days for 12 days by oral
intubation; controls received equivalent amounts of corn oil.

[b]N represents the number of rats in each group.

[c]determinated colorimetrically in liver acid hydrolyzates as described
in the methods.
Values are mean $\pm$ S.E.M.

[d]$P < 0.005$ vs. control.

However, DBT administration at the higher dose of 20 mg/kg produced a greater than two-fold increase ($p < 0.005$) in the amount of hydroxyproline.

Prolyl Hydroxylase Activity

Since prolyl residues are not hydroxylated before they are in peptide-bound form and hydroxyproline does not occur to any significant extent in noncollagen protein, proline hydroxylation can be used as a measure of collagen synthesis in rat liver tissue. The effect of DBT on prolyl hydroxylase activity in rat liver homogenates after 4 and 12 days of treatment is seen in Tables III and IV, respectively. After four days of DBT treatment, prolyl hydroxylase activity increased approximately 50% ($p < 0.01$) in the 10 mg/kg group and about 130% ($p < 0.01$) in the 20 mg/kg group. Animals receiving the same doses of DBT every two days for 12 days (Table IV) showed a relatively modest increase in prolyl hydroxylase activity. There was no change in prolyl hydroxylase activity in the 10 mg/kg group and an increase of approximately 50% ($p < 0.05$) in the 20 mg/kg group.

TABLE III

Effect of Dibutyltin (DBT) on Liver Prolyl Hydroxylase (PH)
Activity in Rats After 4 Days of Treatment [a]

Group	N[b]	PH activity[c] cpm/mg protein
Control	5	1129 $\pm$ 94 (range 857–1297)
DBT (10 mg/kg)	5	1760 $\pm$ 169[d] (range 1326–2226)
DBT (20 mg/kg)	5	2537 $\pm$ 258[d] (range 1709–3095)

[a] DBT in corn oil was administered daily for 4 days by oral intubation; controls received equivalent amounts of corn oil.

[b] N represents the number of rats in each group.

[c] estimated by the formation of ^{3}HHO from incubation at 30^oC of 4-^{3}H-proline-rich substrate and cofactors with 50 and 100 ul aliquots of liver homogenates prepared as described in the methods and reported as cpm ^{3}HHO/mg protein/30 min.
Values are mean $\pm$ S.E.M.

[d] $P < 0.01$ vs. control.

TABLE IV

Effect of Dibutyltin (DBT) on Liver Prolyl Hydroxylase (PH)
Activity in Rats After 12 Days of Treatment [a]

Group	N[b]	PH activity[c] cpm/mg protein
Control	12	2030 ± 202 (range 1290 - 3446)
DBT (10 mg/kg)	15	1873 ± 95 (range 1310 - 2517)
DBT (20 mg/kg)	12	3379 ± 400 (range 1974 - 6527)[d]

[a]DBT in corn oil was administered every 2 days for 12 days by oral
intubation; controls received equivalent amounts of corn oil.

[b]N represents the number of rats in each group.

[c]estimated by the formation of ^{3}HHO from incubation at 30°C of
4-^{3}H-proline-rich substrate and cofactors with 50 and 100 ul
aliquots of liver homogenates prepared as described in the methods
and reported as cpm ^{3}HHO/mg protein/30 min.
Values are mean ± S.E.M.

[d]$P < 0.05$ vs. control.

In Vitro Collagen Synthesis

Although prolyl hydroxylase activity is a sensitive indicator of increased fibrogenesis in liver homogenates, it has not been shown to be a rate-limiting step in collagen biosynthesis. A more direct indication of absolute and relative collagen synthesis is obtained by measurement of in vitro incorporation of ^{3}H-proline into collagenase-degradable and collagenase-resistant protein.

The effect of DBT on in vitro collagen synthesis in rat liver tissue minces after 4 and 12 days of treatment is shown in Tables V and VI, respectively. After 4 days of DBT treatment at a dose of 20 mg/kg, the in vitro incorporation of labeled proline into collagenase-digestible protein increased approximately five-fold ($p < 0.001$). There was a concomitant increase in the amount of label incorporated into collagenase-resistant protein of approximately the same magnitude. As a result, no significant change was observed in the percent of labeled protein digested by collagenase. DBT treatment every two days for 12 days (Table VI) at doses of 10 and 20 mg/kg produced a significant increase in the percent of collagenase-digestible protein at the higher dose only ($p < 0.05$). This increase reflects increased incorporation of labeled proline into collagenase-digestible protein relative to decreased incorporation into collagenase-resistant protein.

TABLE V

In Vitro Collagen Synthesis in Rat Liver Tissue Minces
After 4 Days of Treatment With Dibutyltin (DBT)[a]

Group	N[b]	2,3 ^{3}H-proline released from liver protein by collagenase (cpm/mg protein)[c]	2,3 ^{3}H-proline incorporated into collagenase-resistant protein (cpm/mg protein)[c]	% of total labeled protein digested by collagenase[c]
Control	4	644 ± 226	283,750 ± 164,840	0.098 ± 0.042
DBT (20 mg/kg)	7	3,098 ± 367[d]	1,606,840 ± 185,080[d]	0.039 ± 0.006

[a]DBT in corn oil was administered daily for 4 days by oral intubation; controls received equivalent amounts of corn oil.

[b]N represents the number of rats in each group.

[c]Collagenase digestion performed on protein obtained by precipitation with 5% trichloroacetic acid following 24 hour incubation with labeled proline as described in the methods. Values are mean ± S.E.M.

[d]$p < 0.001$ vs. control.

TABLE VI

In Vitro Collagen Synthesis in Rat Liver Tissue
Minces After 12 Days of Treatment With Dibutyltin (DBT)[a]

GROUP	N[b]	4^3H-proline released from liver protein by collagenase (cpm/mg protein)[c]	4^3H-proline incorporated into collagenase-resistant protein (cpm/mg protein)[c]	% of total labeled protein digested by collagenase[c]
CONTROL	6	307 ± 81	19,200 ± 3,000	0.300 ± 0.081
DBT (10 mg/kg)	7	157 ± 61	11,900 ± 500[d]	0.282 ± 0.100
DBT (20 mg/kg)	5	440 ± 75	13,400 ± 900[d]	0.590 ± 0.082[d]

[a]DBT in corn oil was administered every 2 days for 12 days by oral intubation; controls received equivalent amounts of corn oil.

[b]N represents the number of rats in each group.

[c]Collagenase digestion performed on protein obtained by precipitation with 5% trichloroacetic acid following 24 hour incubation with labeled proline as described in the methods.

[d]$P < 0.05$ vs. control.

Body, Liver and Relative Liver Weights

The effect of four days of DBT treatment (20 mg/kg) on weight gain is shown in Figure I. Dibutyltin-treated rats lost approximately eight grams per day resulting in a mean body weight which was 26% less ($p < 0.001$) than control animals. After DBT treatment every two days for 12 days, the mean body weight was 12% less ($p < 0.05$) than control rats (Table VII).

Histopathology

Dibutyltin treatment at a dose of 20 mg/kg for four days produced inflammatory and biliary tract changes in rats. Inflammatory cells were observed throughout the portal tracts, most notably in and around bile ducts. Proliferation of bile ducts was evident at this time. At a dose of 10 mg/kg for four days, inflammation and biliary damage was minimal.

After 12 days of DBT treatment (20 mg/kg), inflammation and biliary damage was extensive. Both mononuclear and polymorphonuclear cells were present in and around portal tracts and extending out into parenchyma leaving only central vein areas preserved. Bile ducts were proliferated and congested with polymorphonuclear cells; damage to the bile duct epithelial cells was

FIGURE I

Effect of Dibutyltin on Rat Body Weight[a]
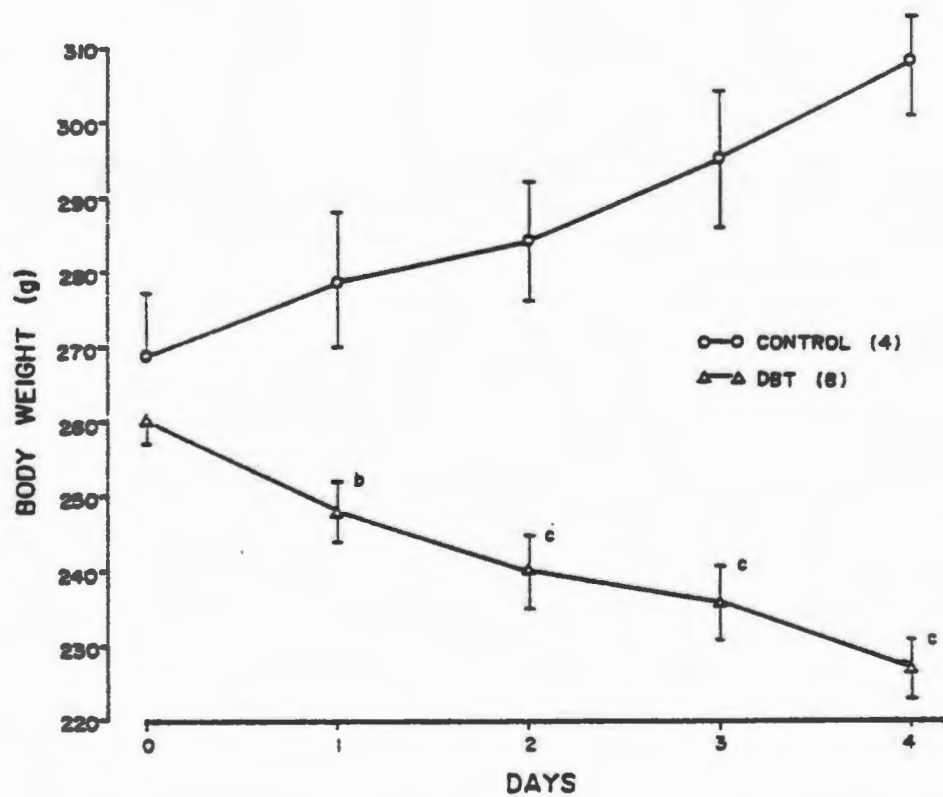
[a]Dibutyltin in corn oil (20 mg/kg) was administered
  every day for 4 days by oral intubation; controls
  received equivalent amounts of corn oil.
  Values are mean - S.E.M.

[b]P < 0.01 vs. control

[c]P < 0.001 vs. control

TABLE VII

Effect of Dibutyltin on Body, Liver and
Relative Liver Weights [a]

Group	N	Body Weight (g)	Body Weight (g)	Relative Liver Weight (g liver/100g body weight)
Control	6	384 ± 14	12.85 ± 0.94	3.33 ± 0.15
DBT (10 mg/Kg)	7	382 ± 6	13.01 ± 0.60	3.40 ± 0.12
DBT (20 mg/Kg)	6	339 ± 14 [b]	13.22 ± 0.70	3.91 ± 0.23

[a] DBT in corn oil was administered every 2 days for 12 days by oral intubation; controls received equivalent amounts of corn oil.

[b] $P < 0.05$ vs. control.

appreciable. Areas of necrosis, both focal and confluent, were seen at this time. Large infarcted areas and non-specific granulomatous lesions were present throughout the parenchyma. Finally, both periportal and parenchymal accumulation of fibrous tissue was demonstrated by trichrome stain. Treatment with 10 mg/kg DBT for 12 days produced moderate inflammation and biliary damage, with minimal fibrosis and necrosis.

DISCUSSION

Biochemical and histological studies of DBT-treated
rat livers after 12 days of treatment reveals increased
accumulation of collagen which is associated with numerous
histopathological changes. Histological observation of
fibrous tissue and the greater than two-fold increase in
hydroxyproline content following 20 mg/kg DBT treatment
indicates the development of hepatic fibrosis which
has not progressed to cirrhosis. There is no increase
in hydroxyproline in the 10 mg/kg group and minimal histo-
logical evidence of fibrous tissue. The correlation
between histologically demonstrable fibrous tissue and
biochemically determined collagen is in agreement with
that seen in dietary (Brunner et al., 1973) and carbon
tetrachloride-induced cirrhosis (Rojkind, 1973).

Biochemical data indicate only a moderate increase
in hepatic collagen synthesis at 12 days when extensive
fibrosis and necrosis are already established by histo-
pathological criteria. The activity of prolyl hydroxylase
is increased only 50% over controls, and there is no
elevation in the amount of collagenase-digestible protein
synthesized in vitro in liver biopsies.

The increased collagen accumulation at 12 days can, however, be attributed to an increase in relative, or percent, total protein synthesis directed toward collagen synthesis as a result of a significant decrease in the synthesis of noncollagen protein. The percent collagen synthesis in vitro is a comparison of collagen and non-collagen synthesis per unit of protein. Histological observation suggests a decrease in viable areas of paren-chyma and an increase in necrotic and fibrous tissue. This in turn would suggest a decrease in noncollagen-producing cells with a resultant increase in collagen production per unit of protein. The two-fold increase in percent collagen synthesized in vitro shown in DBT-treated livers after 12 days supports this conclusion.

Since significant liver hydroxyproline accumulation is evident after 12 days of DBT treatment, the effect of DBT (10 and 20 mg/kg) on collagen synthesis in rat liver is investigated after only four days of treatment. Al-though histological observation of liver biopsies with a collagen specific stain fails to show evidence of fibrosis at four days, biochemical studies demonstrate an increase in collagen synthesis.

Prolyl hydroxylase activity in liver homogenates is increased greater than 50% and 130% in the 10 and 20 mg/kg

DBT-treated rats, respectively. Increased activity of
this enzyme has been shown to precede the increase in
hydroxyproline content in carbon tetrachloride-induced
cirrhosis (Takeuchi et al., 1967) and histological
evidence of advanced cirrhosis in human liver biopsies
(Fuller et al., 1976; Fuller et al., 1977). The observed
increase of prolyl hydroxylase activity in DBT-treated
rat livers, therefore, suggests that fibrogenesis is
increased at four days, prior to observable collagen
accumulation.

Additional evidence for increased collagen synthesis
after four days of DBT treatment is seen in the six-fold
increase in in vitro synthesis of collagenase-digestible
protein. There is no apparent enrichment in the amount of
protein synthesis directed to collagen because of a con-
comitant increase in noncollagen protein synthesis. How-
ever, this increase in noncollagen protein synthesis may
represent the protein made by inflammatory cells detected
by light microscopy.

The effect of DBT on fibroblasts is determined using
monolayer cell cultures of L929 fibroblasts. Prolyl
hydroxylase activity is used as a measure of collagen
synthesis in these cells. A direct correlation between
the activity of this enzyme and the formation of hydroxy-
lated collagen has been reported by Gribble et al. (1969)

in cell cultures of L929 fibroblasts. That incubation
with DBT had no effect on the activity of prolyl hydroxy-
lase in L929 cells suggests that this compound does not
directly stimulate collagen synthesis by fibroblasts
in vivo and that the mechanism of DBT-induced hepatic
fibrogenesis requires a step intermediate to the direct
stimulation of collagen-producing cells.

The increased fibrogenesis seen after four days of
DBT treatment appears to be a sequela of inflammation
and/or bile duct injury and proliferation, the only
histopathological changes observed at this time.

Diethyltin dichloride has been shown to decrease
oxygen uptake in rat brain in vitro by binding to sulf-
hydral groups in α-keto acid oxidases with the resultant
inhibition of these enzymes (Aldridge and Cremer, 1955).
Since the presence of DBT has been demonstrated in the
bile (Barnes and Magee, 1958), and dialkyltin compounds
have many biological properties in common (Stoner et al.,
1954; Barnes and Stoner, 1961), DBT may produce its
biliary damage by decreasing oxygen uptake, thereby pro-
ducing anoxia in bile duct epithelial cells.

Further support for inflammation in portal tracts
and bile duct damage being the primary lesion of DBT is
presented by the histological picture after 12 days of
treatment where the hepatocyte injury observed is

primarily peripheral. At this time, liver injury re-
sembles that of drug-induced hepatitis with necrosis and
fibrosis (Leevy et al., 1976) with the exception of
preserved centrilobular parenchyma.

CONCLUSIONS

(1) After four days of DBT treatment (20 mg/kg),
histological observation of rat livers showed inflammation
in portal tracts and bile duct inflammation and prolifera-
tion. Prolyl hydroxylase activity was increased 50 and
130% over control values in the 10 and 20 mg/kg groups,
respectively. The amount of in vitro collagen synthesis
was increased five-fold in the higher dose group. There
was, however, no change in percent collagen synthesis
since noncollagen protein synthesis was also elevated
five-fold, probably as a result of inflammatory cell
protein synthesis. The data obtained indicate increased
fibrogenesis at four days time although there is no
evidence of fibrosis by histopathological criteria.

(2) After 12 days of DBT treatment (20 mg/kg),
there was extensive mononuclear and polymorphonuclear
cell infiltration in portal tracts, particularly in and
around bile ducts, and within the parenchyma. Unlike
other forms of drug-induced hepatic injury, the central
vein areas were preserved. There were focal and confluent
areas of necrosis, infarcted areas, nonspecific granulo-
matous lesions and an accumulation of fibrous tissue.

Increased collagen content was also reflected by the two-fold increase in hydroxyproline content. Prolyl hydroxylase activity was elevated only 50% over controls and _in vitro_ collagen synthesis was unchanged after 12 days of treatment. However, percent collagen synthesis was increased two-fold.

(3) DBT (3×10^{-7} and 3×10^{-6} M) had no effect on prolyl hydroxylase activity of mouse fibroblasts in cell culture.

(4) These results suggest that DBT-induced fibrogenesis is not a result of direct stimulation of fibroblasts and that biliary damage and/or inflammatory processes are responsible for increased fibrogenesis and associated histopathological changes.

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VITA

Jeannee K. Yermakoff was born on October 28, 1952 in Jersey City, New Jersey. Ms. Yermakoff received her Bachelor of Science degree with honors in Chemistry in 1975. At that time Ms. Yermakoff began her graduate education at the University of Rhode Island in the Department of Pharmacology and Toxicology and completed the requirements for Master of Science degree in May 1978.

During her graduate training, Ms. Yermakoff received Teaching Assistantships from the Department of Pharmacology and the Affirmative Action Program of the University of Rhode Island Graduate School.

Ms. Yermakoff will continue her graduate education at the University of Rhode Island in the Department of Pharmacology and Toxicology where she will work for the Doctor of Philosophy Degree in Pharmacology.