1978

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

Jeannee Karen Yermakoff
University of Rhode Island

Follow this and additional works at: <https://digitalcommons.uri.edu/theses>

Recommended Citation

Yermakoff, Jeannee Karen, "AN EXPERIMENTAL MODEL OF HEPATIC FIBROSIS INDUCED BY THE ADMINISTRATION OF DIBUTYLTIN DICHLORIDE" (1978). *Open Access Master's Theses*. Paper 230.
<https://digitalcommons.uri.edu/theses/230>

This Thesis is brought to you for free and open access by DigitalCommons@URI. It has been accepted for inclusion in Open Access Master's Theses by an authorized administrator of DigitalCommons@URI. For more information, please contact digitalcommons-group@uri.edu.

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
REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
IN
PHARMACOLOGY AND TOXICOLOGY

UNIVERSITY OF RHODE ISLAND

1978

MASTER OF SCIENCE THESIS
OF
JEANNEE KAREN YERMAKOFF

Approved:

Thesis Committee

Major Professor

George C. Fuller
Harold W. Fisher
~~Michael J. ...~~
A. A. Michel
Dean of the Graduate School

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 dialkyltin 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 collagenase-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

The author wishes to express her sincere appreciation to Dr. George C. Fuller for his guidance and inspiration throughout the course of this investigation. Thanks is also extended to the members of her committee and the faculty of the Department of Pharmacology and Toxicology of the University of Rhode Island for their assistance. The help and support of her fellow graduate students, which has been invaluable, is gratefully acknowledged.

Gratitude is extended to Dr. Jesus V. Rodil for his proficient histological evaluation of tissues described herein. The author sincerely thanks Ms. Regina White Santos and Mrs. Nadylis Wood for their skillful technical assistance.

The author wishes to express sincere appreciation to her parents, Serge and Mildred Yermakoff, for their continued support, encouragement and understanding during her academic career.

The author acknowledges the financial support provided by Teaching Assistantships from the Department of Pharmacology and Toxicology and the Graduate School Affirmative Action Program. This investigation was supported in part by a grant awarded to Dr. George C. Fuller from the National Institute on Alcohol Abuse and Alcoholism (AA 01422).

To the Late
Simon Matthew Yermakoff

TABLE OF CONTENTS

ABSTRACT	ii
ACKNOWLEDGEMENTS	iv
DEDICATION	v
TABLE OF CONTENTS	vi
LIST OF TABLES	viii
LIST OF FIGURES	ix
INTRODUCTION	1
LITERATURE SURVEY	3
Collagen Biosynthesis	3
Hepatic Fibrosis in Liver Disease	4
Organotin Toxicity	8
EXPERIMENTAL	12
Animals	12
Materials	12
Cell Culture Techniques	13
Dibutyltin-Induced Hepatic Fibrosis	13
Analytical Procedures	14
Hydroxyproline Determination	14
Preparation of Substrate for Prolyl	
Hydroxylase Assay	15
Prolyl Hydroxylase Assay	16
<u>In Vitro</u> Collagen Biosynthesis	18
Protein Determination	19
Statistical Methods	20
RESULTS	21
Cell Culture Investigation	21
Liver Hydroxyproline Content	21

Prolyl Hydroxylase Activity	24
<u>In Vitro</u> Collagen Biosynthesis	27
Body, Liver and Relative Liver Weights	30
Histopathology	30
DISCUSSION	34
CONCLUSIONS	39
REFERENCES	41
VITA	47

LIST OF TABLES

Table	Page
I Effect of Dibutyltin on Prolyl Hydroxylase Activity in L929 Cells	22
II Effect of Dibutyltin on Liver Hydroxyproline Content in Rats After 12 Days of Treatment . .	23
III Effect of Dibutyltin on Liver Prolyl Hydroxylase Activity in Rats After 4 Days of Treatment	25
IV Effect of Dibutyltin on Liver Prolyl Hydroxylase Activity in Rats After 12 Days of Treatment	26
V <u>In Vitro</u> Collagen Synthesis in Rat Liver Tissue Minces After 4 Days of Treatment with Dibutyltin	28
VI <u>In Vitro</u> Collagen Synthesis In Rat Liver Tissue Minces After 12 Days of Treatment with Dibutyltin	29
VII Body, Liver and Relative Liver Weights	32

LIST OF FIGURES

Figure	Page
I Effect of Dibutyltin on Rat Body Weight . . .	31

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, dialkyltin 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 dibutyltin 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 laboratory models were employed for measurement of hepatic

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 $[\alpha 1(I)]_2 \alpha 2$. 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 glycosylated to galactosylhydroxylysine and glucosylgalactosylhydroxylysine (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 (Lapière 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). Increased 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 (Kershinovich et al., 1970). Fibroblasts were long thought to be responsible for increased collagen biosynthesis 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 collagenase 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 fibrogenesis. 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 complexes in portal tracts preceded the development of cirrhosis in rats induced by immunization with heterologous 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 "Stalidon" disaster in France in 1954 (Barnes and Stoner, 1961). "Stalidon" 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 inflammation 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 libitum.

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.

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 hydroxyproline 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

^aNaCl, 1.285 g; KCl, 0.224 g; MgSO₄, 0.144; CaCl₂, 0.144 g; KH₂PO₄, 0.544 g; NaHCO₃, 2.10 g; D-Glucose, 1.80 g; and distilled water to 1 liter.

4-³H-proline and 20 ul of 1.0 M α, α -dipyridyl, the mixture was incubated for two hours at 37° C under O₂-CO₂ (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 minutes 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 distillation; 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 O₂-CO₂ (95/5%) in 4 mls of Eagles minimum essential medium containing 4-³H- or 2,3-³H-proline (50 uci/ml), 10⁻⁴ M ascorbate and 50 ug/ml β -aminopropionitrile. 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 addition 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% trichloroacetic 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/hydroxyproline 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:

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

b.) Estimated standard error of the mean:

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

c.) t-statistic:

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

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.

TABLE I

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

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

^alate log phase L-929 cells were incubated with DBT for 12 hours.

^bestimated by the formation of ³H₂O from incubation at 30°C of 4-³H-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 ± 0.004 (range 0.105 - 0.133)
DBT (10 mg/kg)	7	0.148 ± 0.019 (range 0.102 - 0.249)
DBT (20 mg/kg)	6	0.266 ± 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 determined colorimetrically in liver acid hydrolyzates as described in the methods.
Values are mean ± 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 + 94 (range 857-1297)
DBT (10 mg/kg)	5	1760 + 169 ^d (range 1326-2226)
DBT (20 mg/kg)	5	2537 + 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 ³HHO from incubation at 30°C of 4-³H-proline-rich substrate and cofactors with 50 and 100 ul aliquots of liver homogenates prepared as described in the methods and reported as cpm ³HHO/mg protein/30 min.
Values are mean ± 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 $\pm$ 202 (range 1290 - 3446)
DBT (10 mg/kg)	15	1873 $\pm$ 95 (range 1310 - 2517)
DBT (20 mg/kg)	12	3379 $\pm$ 400 (range 1974 - 6527) ^d

^aDBT in corn oil was administered every 2 days for 12 days by oral intubation; controls received equivalent amounts of corn oil.

^bN represents the number of rats in each group.

^cestimated by the formation of ³HHO from incubation at 30°C of 4-³H-proline-rich substrate and cofactors with 50 and 100 μ l aliquots of liver homogenates prepared as described in the methods and reported as cpm ³HHO/mg protein/30 min.
Values are mean $\pm$ S.E.M.

^dP < 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 ^3H -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

^aDBT in corn oil was administered daily for 4 days by oral intubation; controls received equivalent amounts of corn oil.

^bN represents the number of rats in each group.

^cCollagenase 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.

^dP < 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	³ H-proline released from liver protein by collagenase (cpm/mg protein) ^c	³ H-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

^aDBT in corn oil was administered every 2 days for 12 days by oral intubation; controls received equivalent amounts of corn oil.

^bN represents the number of rats in each group.

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

^dP < 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

^aDibutyltin 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 $\pm$ 14	12.85 $\pm$ 0.94	3.33 $\pm$ 0.15
DBT (10 mg/Kg)	7	382 $\pm$ 6	13.01 $\pm$ 0.60	3.40 $\pm$ 0.12
DBT (20 mg/Kg)	6	339 $\pm$ 14 ^b	13.22 $\pm$ 0.70	3.91 $\pm$ 0.23

^aDBT in corn oil was administered every 2 days for 12 days by oral intubation; controls received equivalent amounts of corn oil.

^bP < 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 histological 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 histopathological 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 noncollagen synthesis per unit of protein. Histological observation suggests a decrease in viable areas of parenchyma 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. Although 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 concomitant increase in noncollagen protein synthesis. However, 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 hydroxylated 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 hydroxylase 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 sulfhydryl 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 producing 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 resembles 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 proliferation. 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 granulomatous 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.

REFERENCES

- Aldridge, W.N. and Cremer, J.E.: The biochemistry of organotin compounds: diethyltin dichloride and triethyltin sulfate. *Biochem. J.* 61: 406-418, 1955.
- Aldridge, W.N. and Street, B.W.: Oxidative phosphorylation: biochemical effects and properties of trialkyltins. *Biochem. J.* 91: 287-297, 1964.
- Aldridge, W.N. and Street, B.W.: Oxidative phosphorylation: the specific binding of trimethyltin and triethyltin to rat liver mitochondria. *Biochem. J.* 118: 171-179, 1970.
- Aldridge, W.N. and Street, B.W.: Oxidative phosphorylation: the relation between the specific binding of triethyltin and trimethyltin to mitochondria and their effects on various mitochondrial function. *Biochem. J.* 124: 221-234, 1971.
- Aterman, K.: Studies in fibrosis of the liver induced by carbon tetrachloride (1) relation between hepatocellular injury and new formation of fibrous tissue. *Arch. Pathol.* 57: 1-11, 1954.
- Barnes, J.M. and Magee, P.N.: The biliary and hepatic lesion produced experimentally by dibutyltin salts. *J. Path. Bact.* 75: 267-279, 1958.
- Barnes, J.M. and Stoner, H.B.: Toxic properties of some dialkyl and trialkyl tin salts. *Br. J. Ind. Med.* 15: 15-23, 1958.
- Barnes, J.M. and Stoner, H.B.: The toxicology of tin compounds. *Pharmacol. Rev.* 18: 211-231, 1961.
- Bornstein, P.: The biosynthesis of collagen. *Ann. Rev. Biochem.* 43: 567-603, 1974.
- Brunner, G., Perings, E., and Creutzfeldt, W.: D-penicillamine in experimental cirrhosis. *Collagen Metabolism in the Liver.* ed. by H. Popper and K. Becker. Medical Book Corp. Stratton Intercontinental, New York, 1975.

Cameron, G.R. and Karunaratne, W.A.: Carbon tetrachloride cirrhosis in relation to liver regeneration. *J. Path. Bact.* 42: 1-22, 1936.

Chen, T.S.N. and Leevy, C.M.: Collagen biosynthesis in liver disease of the alcoholic. *J. Lab. Clin. Med.* 85: 103-112, 1975.

Chichester, C.O., Fuller, G.C. and Cardinale, G.J.: In vivo labeling and turnover of prolyl hydroxylase and a related immunoreactive protein. *Biochem. Biophys. Res. Commun.* 73: 1056-1062, 1976.

Crystal, R.G.: Lung collagen: definition, diversity, and development. *Fed. Proc.* 33: 2248-2255, 1974.

Diegelmann, R.F., Rothkopf, L.C. and Cohen, I.K.: Measurement of collagen biosynthesis during wound healing. *J. Surg. Res.* 19: 239-243, 1975.

Fujiwara, K., Sakai, T., Oda, T. and Igarashi, S.: The presence of collagenase in Kupffer cells of the rat liver. *Biochem. Biophys. Res. Commun.* 54: 531-537, 1973.

Fuller, G.C., Nolan, J.C., Fink, S., and Rodil, F.V.: Prolyl hydroxylase activity in normal and diseased human liver. *Proc. Soc. Exp. Biol. Med.* 151: 61-64, 1976.

Fuller, G.C., Vidins, E.I. and Rodil, F.V.: Liver prolyl hydroxylase as an indicator of fibrogenesis in alcoholic cirrhosis. *Alcohol and Aldehyde Metabolizing Systems*. eds. R.C. Thurman, J.R. Williamson, H. Drout and B. Chance. Academic Press, New York, 1977.

Gaunt, I.F., Colley, J., Grasso, P., Creasy, M. and Gangolli, S.D.: Acute and short-term toxicity studies on dibutyltin dichloride in rats. *Food. Cosmet. Toxicol.* 6: 599-608, 1968.

Goldberg, B., Taubman, M.B. and Radin, A.: Procollagen peptidase: its mode of action on the native substrate. *Cell* 4: 45-50, 1975.

Gribble, T.J., Comstock, J.P. and Udenfriend: Collagen chain formation and peptidyl proline hydroxylation in monolayer tissue cultures of L929 fibroblasts. *Arch. Biochem. Biophys.* 129: 308-316, 1969.

Guess, W.L. and Haberman, S.: Toxicity profiles of vinyl and polyolefinic plastics and their additives. J. Biomed. Mater. Res. 2: 313-335, 1968.

Guess, W.L. and Stetson, J.B.: Tissue reactions to organotin-stabilized polyvinylchloride catheters. JAMA 204: 118-122 1968.

Huberman, A., Recio, A. and Rojkind, M.: Collagen biosynthesis in normal and cirrhotic rat liver slices. Proc. Soc. Exptl. Biol. Med. 131: 200-2-3, 1969.

Hutton, J.J., Tappel, A.L. and Udenfriend, S.: A rapid assay for collagen proline hydroxylase. Anal. Biochem. 16: 384-394, 1966.

Kent, G., Fels, I.G., Dubin, A. and Popper, H.: Collagen content based on hydroxyproline determinations in human and rat livers: its relation to morphologically demonstrable reticulum and collagen fibers. Lab. Invest. 8: 48-55, 1959.

Kent, G., Gay, S., Inouye, T., Bahy, R., Minick, O.T. and Popper, H.: Vitamin A-containing lipocytes and formation of type III collagen in liver injury. Proc. Natl. Acad. Sci. 73: 3719-3722, 1976.

Kershenovich, D., Ferro, F.J. and Rojkind, M.: The relationship between the free pool of proline and collagen content in human liver cirrhosis. J. Clin. Invest. 49: 2246-2249, 1970.

Kivirikko, K.I., Laitinen, O. and Prockop, D.J.: Modifications of a specific assay for hydroxyproline in urine. Anal. Biochem. 19: 249-255, 1967.

Langness, U. and Udenfriend, S.: Collagen biosynthesis in nonfibroblastic cell lines. Proc. Natl. Acad. Sci. 71: 50-51, 1974.

Lapiere, C.M., Lenders, A. and Kohn, L.D.: Procollagen peptidase: an enzyme excizing the coordination peptides of collagen. Proc. Natl. Acad. Sci. 68: 3054-3058, 1971.

Leevy, C.M., Popper, H. and Sherlock, S. (criteria committee): Diseases of the Liver and Biliary Tract: Standardization of Nomenclature, Diagnostic Criteria and Diagnostic Methodology. Fogarty International Center

Proceedings, No. 22, D.H.E.W. Publication No. (N.I.H.) 76-725, U.S. Govt. Printing Office, Wash, D.C., 1976.

Madden, J.W., Gertman, P.M. and Peacock, E.E.: Dimethyl-nitrosamine-induced hepatic cirrhosis: a new canine model of an ancient human disease. *Surgery* 68: 260-268, 1970.

Magee, P.N., Stoner, H.B. and Barnes, J.M.: The experimental production of edema in the central nervous system of the rat by triethyltin compounds. *J. Path. Bact.* 73: 107-126, 1957.

Miller, E.J.: Biochemical characteristics and biological significance of the genetically-distinct collagens. *Mol. Cell. Biochem.* 13: 165-192, 1976.

Montfort, I. and Perez-Tamayo, R.: The distribution of collagenase in normal rat tissues. *J. Histochem. Cytochem.* 23: 910-920, 1975.

Nimni, M.: Sensitive biological tests for medical grade plastics. I. Toxicity of organotin stabilizers. *J. Pharm. Sci.* 53: 1262-1264, 1964.

Okazaki, I. and Maruyama, K.: Collagenase activity in experimental hepatic fibrosis. *Nature* 252: 49-50, 1974.

Paronetto, F. and Popper, H.: Chronic liver injury induced by immunologic reactions: cirrhosis following immunization with heterologous sera. *Am. J. Path.* 49: 1087-1101, 1966.

Piver, W.T.: Organotin compounds: industrial applications and biological investigation. *Environ. Health. Perspect.* 4: 61-80, 1973.

Popper, H.: Overview of past and future of collagen metabolism. *Collagen Metabolism in the Liver*. ed. by H. Popper and K. Becker, Medical Book Corp. Stratton Intercontinental, New York, 1975.

Popper, H. and Udenfriend, S.: Hepatic fibrosis: correlation of morphological and biochemical investigations. *Am. J. Med.* 49: 707-721, 1970.

Prockop, D.J. and Juva, K.: Synthesis of hydroxyproline in vitro by the hydroxylation of proline in a precursor of collagen. *Proc. Natl. Acad. Sci. U.S.A.* 53: 661-668, 1965.

Rhoads, R.E. and Udenfriend, S.: Purification and properties of collagen proline hydroxylase from newborn rat skin. *Arch. Biochem. Biophys.* 139: 329-339, 1970.

Rojkind, M.: Inhibition of liver fibrosis by L-azetidine-2-carboxylic acid in rats treated with carbon tetrachloride. *J. Clin. Invest.* 52: 2451-2456, 1973.

Rojkind, J. and Diaz De Leon, L.: Collagen biosynthesis in cirrhotic rat liver slices: a regulatory mechanism. *Biochim. Biophys. Acta.* 217: 512-522, 1970.

Seigel, R.C., Pinnell, S.R. and Martin, G.R.: Cross-linking of collagen and elastin. Properties of lysyl oxidase. *Biochemistry* 9: 4486-4492, 1970.

Sinex, F.M., Van Slyke, D.D. and Christman, D.R.: The source and state of the hydroxylysine of collagen II: failure of free hydroxylysine to serve as a source of the hydroxylysine or lysine of collagen. *J. Biol. Chem.* 234: 918-921, 1959.

Speakman, P.T.: Proposed mechanism for the biological assembly of collagen triple helix. *Nature* 229: 241-243, 1971.

Spiro, M.J. and Spiro, R.G.: Studies on the biosynthesis of the hydroxylysine-linked disaccharide unit of basement membranes and collagen. II Kidney galactosyltransferase. *J. Biol. Chem.* 246: 4910-4918, 1971.

Spiro, R.G. and Spiro, M.J.: Studies on the biosynthesis of the hydroxylysine-linked disaccharide unit of basement membranes and collagen. I Kidney glucosyltransferase. *J. Biol. Chem.* 246: 4899-4909, 1971.

Spiro, R.G. and Spiro, M.J.: Studies on the biosynthesis of hydroxylysine-linked disaccharide unit of basement membranes and collagen. III Tissue and subcellular distribution of glycosyltransferases and the effect of various conditions on the enzyme levels. *J. Biol. Chem.* 246: 4919-4925, 1971.

Stone, N. and Meister, A.: Function of ascorbic acid in the conversion of proline to collagen hydroxyproline. *Nature* 194: 555-557, 1962.

Stoner, H.B., Barnes, J.M. and Duff, J.I.: Studies on the toxicity of alkyl tin compounds. Brit. J. Pharmacol. 10: 16-25, 1955.

Takuechi, T., Kivirikko, K.I. and Prockop, D.J.: Increased protocollagen hydroxylase activity in the livers of rats with hepatic fibrosis. Biochim. Biophys. Res. Comm. 28: 940-944, 1967.

Tan, L.P. and Ng, M.L.: The toxic effects of trialkyl-tin compounds on nerve and muscle. J. Neurochem. 29: 689-696, 1977.

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.