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CHARACTERIZATION OF THE FIBROTIC LESION IN DIBUTYLTIN-INDUCED EXPERIMENTAL LIVER INJURY

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CHARACTERIZATION OF THE FIBROTIC LESION IN DIBUTYLTIN-INDUCED EXPERIMENTAL LIVER INJURY

BY

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ABSTRACT

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Administration of di-n-butyltin dichloride (DBT) by oral intubation has been shown to produce hepatic fibrosis in rats. Unlike post-necrotic experimental fibrosis, DBT-induced hepatic fibrosis progresses from inflammation and biliary injury. The relationship of histological changes to various parameters of collagen synthesis and liver injury were investigated. In vitro collagen synthesis was determined by incubation of liver biopsies with labelled proline and subsequent digestion with protease-free collagenase. Prolyl hydroxylase activity in liver homogenates was taken as a second parameter reflecting the rate of collagen synthesis. DBT administration (20 mg/kg/day, p.o.) produced a progressive increase in both in vitro collagen synthesis and prolyl hydroxylase activity from 0 to 4 days. The increases in these two parameters were significantly correlated and both accompanied the appearance of inflammatory cells in portal tracts and bile duct proliferation. Serum alanine transaminase activity was increased after 4 days of DBT administration. DBT administration (20 mg/kg, q.o.d., p.o.) for 12 days produced increased prolyl hydroxylase activity, an increase in the percent of in vitro collagen synthesis, and the accumulation of hydroxyproline in the liver. These biochemical changes were associated with fibrosis, bile duct proliferation and extensive inflammation in the portal tracts. Alanine transaminase and alkaline phosphatase activities

in serum were unchanged after 12 days of DBT treatment. Rats 4 weeks of age at the start of treatment were relatively resistant to the effects of DBT (20 mg/kg, q.o.d., p.o.) administered for 12 days. Prolyl hydroxylase activity in these 4-week old rats was 125% of control compared to 150% and 215% in the 9- and 14-week old groups, respectively. Leucocytosis and weight loss usually associated with DBT-induced liver injury were absent in these younger rats and histology was similar to that of control animals. Thymus atrophy was observed in the 4-week old rats. Investigation of lymphocyte-mediated cytotoxicity, delayed type hypersensitivity, and immunofluorescence in rats 9 weeks of age at the start of treatment failed to reveal an immunologic reaction against DBT or liver. The relationship of differences in hepatic microsomal metabolism to the age-related phenomena was investigated using an inhibitor of microsomal enzymes, SKF 525A. Administration of SKF 525A (50 mg/kg, b.i.d., p.o.) concomitantly with DBT (20 mg/kg/day, p.o.) resulted in an interaction between these two compounds with respect to their effect on prolyl hydroxylase activity. The elevation in serum alanine transaminase activity seen with DBT treatment alone after 4 days was completely prevented by the simultaneous administration of SKF 525A. Periportal inflammation and biliary changes were absent in rats receiving DBT and SKF 525A.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The toxicity of organotin compounds became apparent in the mid 1950s following their unfounded use as therapeutic agents and ensuing disastrous results. The toxicology of organotin derivatives remains of interest today as a result of their continued industrial uses. Alkyltins are widely used as stabilizers and catalysts by the plastics industries (Piver, 1973) and as biocidal agents (McCollister and Schober, 1975).

The hepatic and biliary manifestations of dialkyltin toxicity in rats has been known for some time (Barnes and McGee, 1958; Barnes and Stoner, 1958; Gaunt et al., 1968). More recently, toxicity to thymus-dependent lymphocytes *in vitro* and impairment of cell-mediated immune functions in rats have been attributed to the dialkyltins (Seinen et al., 1977a and 1977b).

Administration of di-n-butyltin dichloride (DBT) to rats has been shown to produce an experimental model of hepatic fibrosis which is associated with periportal inflammation and bile duct proliferation (Yermakoff, 1978). This investigation attempts to further characterize the fibrotic hepatic lesion induced by this organotin compound. The relationship of DBT-induced changes in biochemical parameters of hepatic collagen synthesis and liver injury to the associated histopathology is examined with special emphasis on hepatic inflammation.

A possible connection between this compound's hepatotoxic and immunotoxic properties is investigated. The role of metabolism in DBT-induced liver injury is also examined.

LITERATURE SURVEY

Collagen Biosynthesis

Collagen is the most ubiquitous protein in higher vertebrates. Its presence in the extracellular matrix, as rigid fibers consisting of cross-linked collagen molecules, provides structural support in these organisms. Collagen molecules each consist of three alpha (α) chains, left-handed helices, coiled into a unique right-handed triple helix.

At least five, and possibly more, types of collagen molecules have been identified; these have been recently reviewed by Eyre (1980). Intersitial collagens include types I and III. Type I, found primarily in skin, bone, and tendon, consists of two $\alpha 1$ chains and one $\alpha 2$ chain, which are distinguishable by their separation on carboxymethylcellulose columns. It is represented by the molecular formula $[\alpha 1(I)]_2\alpha 2$. Types II and III each consist of three identical $\alpha 1$ chains, with each type differing slightly in amino acid composition. Type II ($[\alpha 1(II)]_3$) is found in cartilage and type III ($[\alpha 1(III)]_3$) in fetal tissue, uterus and blood vessels. Collagen of the basal lamina, basement membrane collagen, is distinct from the previous three in its lack of fibrillar structure. Its precise molecular structure, originally thought to be $[\alpha 1(IV)]_3$, is under question. Recently, collagen αA and αB chains have been isolated from various sources including human placenta, infant cartilage (Rhodes and Miller, 1978), and lung parenchyma (Madri and Furthmayr, 1979). Their molecular formula(s) is uncertain.

Collagen is unique among proteins in the extent of its post-translational modifications. Its biosynthesis consists of the assembly of a proline-, lysine-, and glycine-rich polypeptide precursor, procollagen. Hydroxylation of both proline (Prockop and Juva, 1965) and lysine (Sinex et al., 1959) has been shown to occur subsequent to the incorporation of these amino acids into procollagen chains. Intracellular hydroxylation of collagen precursor has been shown to be requisite for stable helix formation and secretion (Uitto and Prockop, 1974). Following hydroxylation of lysine, some of these residues are glycosylated to galactosylhydroxylysine and glucosylgalactosylhydroxylysine (Spiro and Spiro, 1971a and 1971b). Cellular processing of procollagen α chains also includes interchain disulfide-bond linkage in nonhelical regions of the molecule which is essential for triple-helix formation (Speakman, 1971).

Following the secretion of procollagen, terminal nonhelical regions are enzymatically removed (Tuderman et al., 1978; Davidson et al., 1977). Collagen cross linking occurs through additional extracellular processing by the enzyme lysyl oxidase, which oxidatively deaminates specific lysine residues leaving aldehyde moieties. These, in turn, form cross links through condensation reactions (Siegal et al., 1970).

In mammals, collagen is cleaved by collagenases to form two fragments, referred to as TC_A and TC_B, which are then degraded further by relatively nonspecific proteases (Perez-Tamayo, 1978). The enzyme probably exists in vivo in three forms: latent enzyme, free enzyme, and enzyme bound to collagen.

Inflammatory and Immunologic Processes in Hepatic Fibrosis

It is well-established that the amount of collagen is increased in liver disease resulting from exposure to various xenobiotics. Such fibrosis is associated with chronic alcoholism (Chen and Leevy, 1975; Mann et al., 1979), exposure to environmental toxicants (Popper et al., 1979), and certain therapeutic agents (Maddrey and Boitnott, 1979). Xenobiotic-induced liver disease has been broadly classified into three categories: cytotoxic, which include steatosis; cholestatic; and mixed cytotoxic and cholestatic (Zimmerman, 1978).

Regardless of etiology and histologic classification, all types of chemical-induced hepatic injury share the possibility of progression to fibrosis via one or a combination of the following processes. The first of these is the stimulation of collagen production by or increased proliferation of collagen-producing cells. The second is the increased extracellular maturation of collagen fibrils from secreted procollagen. A decrease in collagenolytic processes may result in fibrosis. Finally, a minor contribution to fibrosis may result from the collapse and aggregation of preformed collagen fibers.

The mechanism of chemical-induced hepatic fibrogenesis has been extensively investigated utilizing models of fibrosis induced by compounds such as carbon tetrachloride (Aterman, 1954; McLeon et al., 1969; McGee et al., 1973) and dimethylnitrosamine (Madden et al., 1970), which are predominately post-necrotic. These studies have led to the hypothesis that hepatocytes, upon their death, release products to

stimulate fibrogenesis (Popper, 1975). However feasible, the application of this hypothesis to most types of xenobiotic-induced liver fibrosis in humans is somewhat untenable. Specifically, alcoholic and drug-induced fibrosis in humans is commonly associated with an imflammatory and/or immunologic component not seen in these post-necrotic models of hepatic fibrosis.

Inflammatory and/or immunologic processes[a] have been associated with a broad range of fibrotic diseases. Fibrogenesis in inflammatory/immunologic disease states has been attributed to fibroblast proliferation (Wahl et al., 1978), increased collagen synthesis by fibroblasts (Johnson and Ziff, 1976), and fibroblast chemotaxis (Posthelwaite et al., 1976) in response to factors derived from immunocompetent cells.

Recently, evidence has been accumulating that immunocompetent cells and products released by these cells play a substantial role in the initiation and/or perpetuation of hepatic fibrogenesis. In both hepatitis B (HB) antigen-negative and HB antigen-positive hepatitis, autoimmune aggression has been implicated as a means of perpetuating liver cell injury (Paronetto and Popper, 1976). In a recent review, Buschenfelde et al. (1979) suggested that the HB virus by itself is not injurious to cells and that the host immune response is responsible for the tissue damage in acute and chronic HB virus-induced hepatitis. In HB antigen-negative hepatitis, antibody against native liver membrane antigens has been detected, which corresponds with histologic evidence

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[a]Unless otherwise specified, throughout this text these processes are considered together since it is often difficult to clearly distinguish one from the other. Inflammation may be a component and/or consequence of an immune response (Voisen, 1977).

of inflammation and continuing necrosis (Hopf et al., 1976). A cell-mediated immune response to collagen has been suggested to initiate the progression of hepatitis to cirrhosis (Chen et al., 1978). In primary biliary cirrhosis as many as one-third of patients have been estimated to possess antiductular cell antibodies and 80% antimitochondrial antibodies (Schaffner, 1979). Finally, experimental liver injury has been produced in rats by immunization with heterologous sera (Paranetto and Popper, 1966).

Inflammatory and/or immunologic processes are part of the pathogenetic scenario of drug- and chemical-induced hepatic fibrosis in many instances. However, it is difficult to determine the relative contribution of these processes to the etiology and perpetuation of such liver disease. The primary reaction may be an inflammatory and/or immunologic response to the drug and/or drug products, a toxic reaction to the compound and/or its metabolites, or any combination of these phenomena (Maddrey and Boitnott, 1979).

Understanding of immunologic reactivity to drugs and other chemicals, or drug allergy, has been advanced by recent work with penicillamine. This drug has been shown to react with compounds containing disulfide groups to form disulfides which are at least partially responsible for the immune response resulting from penicillamine administration (Dewdney, 1979). Such work has suggested that the potential for drug allergy is a function of a compound's ability to form covalent bonds with macromolecules at physiological conditions (McGovern et al., 1970; Dewdney, 1979). The knowledge of the formation of haptenic determinants has been of some predictive value in drug allergy, but

unequivocal demonstration of cause and effect has been hampered by the diversity of immunologic responses and methodological inadequacies (DeWeck, 1979).

The association of drug with disease in suspected hepatic manifestations of drug allergy has been even more elusive. When immune reactivity to a drug can be demonstrated, it does not necessarily imply that such reactivity is the cause of liver injury. Consequently, the nature of immunologic reactions involved in hepatic drug allergy have not been clearly identified (Girard, 1974).

Hepatic manifestations of drug and chemical allergy are commonly diagnosed by indirect means such as recurrence of symptoms with repeated exposure. The major problem resulting from this practice is the identification of host idiosyncracy as drug allergy. Nonetheless, much of the evidence has been convincing. Halothane produces jaundice in a small number of patients (Zimmerman, 1972), and, in a remarkably thorough case study, repeated episodes of hepatitis have been correlated with exposure to halothane in an anesthetist (Klatskin and Kimberg, 1969). Primary biliary cirrhosis, a disease in which the majority of patients demonstrate autoantibodies, is often associated with a history of drug allergy (Paronetto et al., 1964a); phenothiazines and androgenic steroids produce liver injury similar to that seen in primary biliary cirrhosis (Dickson et al., 1979). The environmental toxicant, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), produces liver injury (Neal et al., 1979) and is suspected to be immunogenic (Luster et al., 1979) in mice. However, a connection between chemical-induced experimental liver injury and antigenicity of such toxicants has not been established.

Drugs and other chemicals may also produce a destructive cholangitis in which inflammatory processes occur in the liver in the absence of evidence of immunologic phenomena (Zimmerman, 1978). Such inflammation has been suggested to be a reaction to cholestasis (Popper and Schaffner, 1970) although cholestasis is not always the primary event (Krstulovic, 1968). The distinction between such inflammatory reactions and immunologic processes is not well defined. In fact, it has been suggested that, in many cases, cholangitis is responsible for subsequent immunologic reactions (Popper and Schaffner, 1970).

Developmental Aspects of Hepatic Drug Metabolism

Clinical problems associated with pharmakokinetic differences between the perinatal period and postnatal development through adulthood have become increasingly apparent in recent years. As always, there are exceptions. Yet, it is common for plasma half lives to be up to a magnitude greater in the neonate, apparent volumes of distribution to be substantially increased, and various drug metabolism processes to be sluggish compared to adulthood (Done et al., 1977). Unless dosage is adjusted accordingly, substantial toxicity is likely to occur in children as a result of extraordinarily high plasma drug levels.

Investigation of developmental aspects of pharmacokinetic parameters in animals has largely been supportive of human data. Although changes in drug disposition and excretion probably contribute (Niems et al., 1976), pharmakokinetic differences during development are largely a result of differences in drug metabolism. Striking variations

occur during rat development in both biosynthetic reactions such as glucuronidation (Dutton, 1978) and oxidative reactions of the cytochrome P450 monooxygenase system (Niems et al., 1976). This review will concern itself with the latter.

Although the pattern of variations in monooxygenase activity in the liver varies with substrate, sex, and species, certain generalizations can be made. Activity of microsomal enzymes usually begins to increase at birth, after which it either continues to rise, reaching a plateau at maturity, or peaks, returning to a plateau with maturation (Kato and Takanaka, 1968; Niems et al., 1976). Although the metabolism of a few drug substrates, such as the hydroxylation of aniline in the rat, peaks as early as two weeks postnatal (Gram et al., 1969), others, such as the N-demethylation of aminopyrine in the rat, peak as late as nine weeks (Henderson, 1971). Somewhere during three to eight weeks postnatal, adult levels of P450 monooxygenase capability is reached for most substrates (Gilette and Stripp, 1975).

Various mechanisms have been proposed for decreased oxidative drug metabolism in young animals. Livers removed from rats injected with somatotropin demonstrated impaired in vitro microsomal metabolism of various drug substrates (Wilson, 1969). Since levels of growth hormone decrease postnatally, somatotropin may play a permissive role in the developmental variations in drug metabolism. Pelkonen (1973) suggests that "endogenous ligands" in fetal liver contribute to decreased drug oxidations following the observation that binding spectra of aminopyrine in fetal liver are the inverse of the characteristic type I spectra.

Biliary Excretion

Biliary excretion has been shown to be a major route for the elimination of xenobiotics from the body in both animals and humans (Stowe and Plaa, 1968). Bile production arises from the active secretion of bile salts by hepatocytes, which is accompanied by the secretion of water and electrolytes (Levine, 1978). Three apparently distinct transport processes have been recognized for the excretion in the bile of cationic, amnionic, and polar, but uncharged, molecules all with a minimum molecular weight somewhere between 200-300 (Levine, 1978). Subsequent to biliary excretion, xenobiotics may be excreted in the feces or, those with appropriate chemical properties, may be reabsorbed to enter the enterohepatic circulation. Compounds which enter this circulation tend to have prolonged half lives which may contribute to toxicity. In addition, this circulation has been shown to be prerequisite for the development of certain toxic reactions. Thompson et al. (1954) found that chloramphenicol, subsequent to hepatic metabolism and biliary excretion, is converted to a toxic metabolite in the gut and reabsorbed as such to produce thyroid hypertrophy and resultant metabolic disturbances.

Substantial species variation has been observed in the biliary excretion of some compounds. Russell and Klaassen (1972) have shown that the bile/plasma concentration gradient of ouabain following administration to rats, rabbits, and dogs was four orders of magnitude greater in the rat than the latter two species. Observed differences in the monkey and rabbit in the biliary excretion of disodium chromo-

glycate, a drug not subject to hepatic metabolism, suggests that species variation of xenobiotic excretion in the bile in not necessarily due to interspecies metabolic differences (Ashton et al., 1973).

Hepatic metabolism plays a role in the biliary excretion of many xenobiotics. The contribution is most pronounced when compounds are increased in molecular weight and/or acquire a polar group through metabolism without which biliary excretion would not occur. This has been demonstrated in rats with compounds such as biphenyl (Millburn et al., 1967) and orphenadrine (Hespe and Kafoe, 1970). In both cases, the parent compound is not excreted in the bile unchanged although its hydroxylated and glucuronidated metabolites are excreted extensively.

Decreased or absent bile flow occurs in cholestasis. The condition presents itself morphologically as bile plugs (Popper and Schaffner, 1970) and biochemically as an elevation of serum alkaline phosphatase due to induction of this enzyme (Kaplan and Righetti, 1970). Cholestasis may be either extra- or intrahepatic, the former being associated with an obstruction such as a tumor or inflammatory lesion outside the liver. In either case, a common disturbance of the secretion of bile salts and lipid micelles is thought to exist (Popper and Schaffner, 1970).

Organotin Toxicity

Extensive investigation of the toxicity of organotin derivatives began in the mid 1950s. The impetus for this was the "Stalinon" disaster in France in 1954. The available information regarding the

incident is reviewed by Barnes and Stoner (1959). "Stalinon" was the propriety name for a drug sold for the treatment of skin infections. It was said to contain 15 mg/capsule of diethyltin diiodide. According to the recommended dosage, patients should have received approximately 90 mg/day of this organotin; in reality, they ingested approximately 80 mg/day of diethyltin and 10 mg/day of the contaminant triethyltin. Patients exhibited symptoms of both central nervous system and gastrointestinal toxicity, which resulted in approximately 100 deaths.

In the 25 years since "Stalinon", investigations of organotins have revealed that both diversified and relatively potent toxicity results from these compounds; reviews are available on the subject (Barnes and Magos, 1968; McCollister and Schober, 1975; Kimbrough, 1976).

Organotin derivatives have been adopted for a variety of industrial uses in recent years. They have been used as stabilizers in the production of polyvinyl chloride; catalysts in the making of polyurethane (Piver, 1973); and as miticides, fungicides, and anthelmintics (McCollister and Schober, 1975; Hunter, 1976). Since many of these biocides ultimately may contaminate food products, acceptable daily intakes have been established for organotin pesticides by the FAO/WHO Joint Expert Committees on Pesticide Residues (McCollister and Schober, 1976). The values established range from 0.5 to 7.5 μg/kg/day, quite a constrast to the accepted use of organotin compounds a little more than two decades ago.

The trialkyltins produce central nervous system toxicity, which has been attributed to reversible interstitial edema of the white

matter of the brain (Magee et al., 1957). Inhibition of oxidative phosphorylation (Aldridge and Street, 1964) and swelling of liver mitochondria (Wulf and Byington, 1975) have been observed in vitro in rat liver mitochondria with trialkyltin derivatives. 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). Tributyltin undergoes oxidative metabolism by the rat liver microsomal system (Kimmel et al., 1977) to form metabolites which retain much of the biologic activity of the parent compound (Aldridge and Street, 1977) or unstable metabolites which are rapidly destannylated.

Dialkyltin compounds differ from their trialkyl counterparts in that the primary lesion of the former is biliary and hepatic injury (Barnes and Stoner, 1958; Barnes and Magee, 1958; Gaunt et al., 1968). Dibutyltin is the most active in this respect. Barnes and Magee (1958) reported bile duct injury and hepatic inflammation two days after oral administration of dibutyltin dichloride (50 mg/kg). After six months, hepatic fibrosis was evident.

In addition to its effects on the liver, Middleton and Platt (1977) report an inflammatory reaction in rat skin following the application of dibutyltin. Contact dermatitis from elastic fabrics with a vinyl chloride polymer component has been attributed to the presence of a dibutyltin stabilizer (Fisher, 1973).

Seinen et al (1977a) have reported atrophy of the thymus, spleen, and popliteal lymph node in weaning rats and toxicity to rat thymocytes

in vitro from dibutyltin. Thymocyte-dependent immunity in rats was found to be impaired by dibutyltin administration (Seinen *et al.*, 1977b). Allograft rejection was delayed, and the response toward t-depdendent antigen, but not t-independent antigen, was decreased. These effects were greater in rats treated pre- and postnatally.

Dialkytin's toxic effects have been attributed to their ability to inhibit α-keto acid oxidases in liver mitochondria and brain brei of the rat (Aldridge and Cremer, 1955); inhibition of oxidative phosphorylation by binding to the adenosine-triphosphatase complex as seen in ox heart mitochondria (Cain *et al.*, 1977); and impairment of amino acid transport by binding to membrane sulfydral groups in rat thymocytes (Miller *et al.*, 1978).

Kimmel *et al.* (1977) have shown that dibutyltin derivatives undergo hepatic microsomal monooxygenase metabolism in rats, at least in part. Metabolism was investigated by Kimmel *et al.* in rat liver microsomal system and *in vivo* in mice using ^{14}C-dibutyltin diacetate. Unidentified dialkyl polar metabolites of dibutyltin were suggested to be butyl-hydroxylated derivatives. The lesser amount of monoalkyltin recovered was thought to result from both nonenzymatic destannylation and decomposition of unstable hydroxylated dibutyltin derivatives. The majority of labelled dibutyltin administered to mice was recovered in the feces between 42-90 hours suggesting biliary excretion. Other investigators have reported the concentration of dibutyltin in the bile to be slightly higher than plasma levels 18 hours after oral administration in rats (Barnes and Magee, 1958).

Naproxen

Naproxen (6-methoxy-α-methyl-2-napthaleneacetic acid) was synthesized by Harrison et al. (1970) in an attempt to provide an improved antirheumatic drug, an effort which has proven to be successful (Muirden, 1978). Naproxen is reported to be more than ten times as potent as aspirin in preventing carrageenin-induced rat paw edema, a commonly used test for antiinflammatory activity (Roszkowski et al., 1971). Its usefulness is also a function of its relatively long half life in animals and humans (Runkel et al., 1972) as compared to other nonsteroidal antiinflammatory drugs.

Roszkowski et al. (1971) suggest that naproxen acts directly at the site of inflammation. Although some nonsteroidal antiinflammatory drugs have been reported to produce their pharmacological effects at least partially by glucocorticoid agonist activity, naproxen does not appear to possess such properties (Feldman, 1978). Naproxen's effects in vivo are more likely to be related to its inhibition of prostoglandin synthesis (McIntyre et al., 1978).

SKF 525A

SKF 525A (β-diethylaminoethyl-diphenyl-n-propylacetate) has been shown to inhibit hepatic microsomal metabolism in vivo (Stitzel et al., 1966), in vitro (Anders and Mannering, 1966a), and in an extracorporeal system (Stitzel et al., 1968). Anders and Mannering (1966a) suggest that SKF 525A produces its effects by competitve inhibition with drug substrates. However, other investigators have found both noncompetitive

and competitive inhibition of the same drug substrate using SKF 525A before and after recrystallization with benzene (Jenner and Netter, 1972). No explanation was offered for the effect of recrystallization; the structure of SKF 525A was unchanged.

SKF 525A is not as meticulous in its inhibition of hepatic microsomal metabolism as originally suspected. It inhibits primarily oxidative reaction in liver microsomes, but inhibition of some glucoronidations has been reported (Levine et al., 1970). Its inhibitory effects do not extend to the metabolism of all drug substrates (Goldstein et al., 1974). In addition, its inhibition of drug metabolism appears to exhibit temporal dependence (Anders and Mannering, 1966b). Rats treated with SKF 525A for five days (50 mg/kg/day, i.p.) showed increased hexobarbital metabolism 24 hours after the last dose of inhibitor, and effect which was attributed to induction of microsomal enzymes. However, when these rats were given a sixth dose of SKF 525A, they exhibited significant inhibition of hexobarbital metabolism 135 minutes later. Based on these results, these investigators suggest that SKF 525A maintains its inhibitory properties with both control and SKF 525A-induced microsomal enzymes.

SKF 525A has pharmacological effects in addition to those it exerts on drug metabolism. Infusion of this inhibitor into the renal artery of dogs at concentrations of 4 mg/minute and 8 mg/minute increased urine volume and sodium excretion, respectively (Marshall and Williamson, 1964). Magus and Fouts (1967) reported that SKF 525A has variable effects on tryptophan pyrrolase including induction and "deattenuation"

of hydrocortisone induction of this enzyme in rat liver. This inhibitor has also been reported to impair gastrointestinal absorption of drugs in rats (McLean and Marchand, 1970).

Biliary excretion of stilbestrol, phenolphthalein (Levine et al., 1970), and 3-methylcholanthrene (Levine, 1972) was decreased in rats as a result of SKF 525A administration. This effect was thought to occur as a result of SKF 525A's effect on metabolism of these drugs since this inhibitor had no effect on the biliary excretion of administered phenolphthalein glucuronide (Levine et al., 1970). Hespe and Kafoe (1970) investigated the possibility that SKF 525A, or a metabolite, produced its effects on biliary excretion by competition for elimination in the bile. Following the finding that SKF 525A enhances biliary excretion of labelled orphenadrine, they tested the effects of pretreatment with unlabelled orphenadrine and found that excretion of the labelled compound was unchanged. This suggests that SKF 525A does not enhance elimination of compounds in the bile by competition with a saturable transport process.

EXPERIMENTAL

Materials

Di-n-butylin dichloride was purchased from Aldrich Chemical Company (Milwaukee, WI). Naproxen (NAPROSYN) was a product of Syntex Laboratories (Palo Alto, CA). SKF 525A was provided by Smith, Kline, and French Laboratories (Philadelphia, PA).

The following radioactive materials were purchased from New England Nuclear (Boston, MA): 2,3-^{3}H-proline (specific activity 20 Ci/mmole), 4-^{3}H-proline (specific activity 25-30 Ci/mole) and ^{51}Cr-sodium chromate (specific activity 200-500 Ci/g).

Reagents purchased from Sigma Chemical Company (St. Louis, MO) included 2,3-dimercaptopropanol, 2-amino-2-methyl-1-propanol, p-nitrophenol, and p-nitrophenylphosphate. Dulbeccos Modified Eagles Medium (DME), RPMI 1640, and phytohemagglutinin were products of Grand Island Biologicals (Grand Island, NY). Concanavalin A was a product of Calbiochem (La Jolla, CA). FITC-conjugated anti-rat immunoglobulinG (IgG) was purchased from Miles Yeda, Ltd. (Rehovat, Israel). Eastman Kodak (Rochester, NY) was the supplier of 2,4-dinitrophenyl hydrazine. All reagents used throughout the investigation were analytical grade.

Animals

Male CD-Crl:CD(SD)Br rats were purchased from Charles River

Breeding Laboratories (Wilmington, MA). The rats were housed in groups of four to six in colony cages at an ambient temperature ranging from 24-27°C with alternating 12-hour light/dark cycles. Rats were fed Charles River Rat-Mouse-Hamster Formula and water ad libitum.

Rats were 9 weeks of age at the start of treatment in all studies unless specified otherwise.

Dibutyltin-induced Hepatic Fibrosis

Fibrosis and associated liver injury were produced in rats by the oral administration of di-n-butyltin dichloride (DBT) in corn oil at a dose of 20 mg/kg. Unless otherwise specified, 0 to 4 day DBT administration was on a daily basis and 12-day treatment on alternate days. Between 20-24 hours after the last administration of DBT, rats were killed by decapitation or, where serum assays were performed, by ether anesthesia and subsequent exsanguination. Livers, and sometimes thymuses and spleens, were removed and quickly chilled. Sections of liver tissue, and on occasion feet, were fixed and prepared for light microscopy with hematoxylin-eosin and trichrome stains. Liver samples were also prepared for determination of prolyl hydroxylase activity and in vitro collagen synthesis. Blood was added to vials containing 8.2 mg disodium EDTA in 0.1 ml water for leucocyte counts or kept on ice for approximately 30 minutes and centrifuged at 3000 rpm to obtain serum.

Additional Animal Treatments

Naproxen was administered at a dose of 15 mg/kg every 12 hours by

oral intubation for 12 days. It was prepared as a 7.5 mg/ml suspension in corn oil. Controls received equivalent amounts of vehicle.

SKF 525A was injected intraperitoneally at a dose of 50 mg/kg in saline every 12 hours for 4 days. Controls received equivalent amounts of saline.

All drugs, regardless of the route of administration, were given in a volume of less than 1 ml.

Preparation of Substrate for Prolyl Hydroxylase Assay

The tritium labelled substrate was prepared by the method of Hutton et al. (1966) with some modifications. Twenty-five to 30 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 6 gram portions of minced embryo were placed in 50 ml beakers and enough Krebs buffer was added to bring the final volume to 20 ml. Following the addition of 1.0 mCi of 4-^{3}H proline and 20 µl of 1.0 M α,α-dipyridyl in ethanol, the mixture was incubated for 2 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/g tissue) overnight at 4°C. After centrifugation for 90 minutes at 30,000 rpm,

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[a] NaCl, 1.285 g; KCl, 0.224 g; $MgSO_4$, 0.144 g; $CaCl_2$, 0.1444 g; KH_2PO_4, 0.544 g; $NaHCO_3$, 2.10 g; D-glucose, 1.80 g; and distilled water to 1 liter.

the supernate containing newly-formed, unhydroxylated collagen was dialyzed against 10 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 40-50 mg aliquots of tissue were homogenized in 600 µl of buffer containing 0.25 M sucrose, 10^{-5} M disodium EDTA, 10^{-5} M dithiothreitol, and 0.1% Triton. Prolyl hyroxylase activity in 50 and 100 µl 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 collagen substrate (0.1 ml), 1.0 mM ascorbate, 0.1 mM α-ketoglutarate, 0.1 mM ferrous ammonium sulfate, 0.05 M Tris CHl buffer (pH 7.4), 0.2% bovine serum albumin and 0.04% catalase. The prolyl hydroxylase reaction was stopped after 30 minutes by the addition of 0.1 volume 50% trichloracetic 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 labelled substrate as described above (Rhoads and Udenfriend, 1970). Due to variability in substrate batches, a conversion factor, derived from substrate calibration with standard enzyme, was employed to permit comparisons of prolyl hydroxylase activity values when different substrate preparations are used. Values were then converted to International Units which are defined as "that quantity of enzyme that will catalyze the reaction of one μmole of substrate per minute" (Kachmar and Moss, 1976).

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 20 mg of freshly dissected liver minces were incubated for 24 hours at 37°C under O_2-CO_2 (95/5%) in 4 ml of Dulbecco's Modified Eagles medium (DME) containing 2,3-^{3}H-proline (50 μCi/ml), 0.56 mM ascorbate, and 0.71 mM 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 (Mann *et al.*, 1979). The incubation mixture was frozen, thawed, and homogenized in a Bellco glass/glass homogenizer. It was then dialyzed against 100 volumes of 0.5 M acetic acid, containing 10^{-5} M disodium EDTA, with two changes and against 100 volumes of 0.05 M Tris HCl buffer (pH 7.4),

containing 2.5 mM n-ethylmaleimide, with two changes to remove unincorporated labelled proline. Tubes of 0.4 ml aliquots of sample were then incubated for 18 hours at 37°C in a mixture containing 0.05 Tris HCl buffer (pH 7.4), 2.5 mM n-ethylmaleimide, 0.5 mM calcium chloride, 0.1 mL chloroform and 130 units of bacterial collagenase in a final volume of 1.0 ml. Duplicate tubes were incubated without collagenase. 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 ml Digestol^{R} (Yorktown, Hackensack, NJ) and counted in 10 ml Hydromix^{R} scintillation cocktail (Yorktown, Hackensack, NJ). The 5% trichloroacetic acid supernate was again coprecipitated using bovine serum albumin (1.0%) and counted in 10 ml Hydromix^{R}. Protein content was determined on liver homogenates. Collagen and noncollagen protein synthesis per unit protein were calculated from collagenase-digested protein in the supernate and collagenase-resistent 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 as follows (Diegelmann et al., 1975):

$$\%\ \text{collagen synthesis} = \frac{\text{cpm in supernate}}{(\text{cpm in pellet} \times 5.4) + (\text{cpm in supernate})} \times 100$$

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 mμ on a Gilford 250 Sprectrophotometer.

Serum Alanine Transaminase

Serum alanine transaminase was determined by the method of Reitman and Frankel (1957). Serum (0.2 ml) was incubated at 37°C for 30 minutes with 1.0 ml substrate containing 2.0 μmoles/ml α-ketoglutarate and 200 μmoles/ml alanine. One ml of 2,4-dinitrophenylhydrazine (1.0 μmole/ml) was added to each tube and samples were left at room temperature for 20 minutes after which 10 ml of 0.4 M NaOH was added. Following 30 minutes for color development, absorbance at 520 mμ was determined. Both reagent and serum blanks were prepared and all samples compared to a standard curve ranging from 0-1.0 μmoles of pyruvate. Values were then converted to International Units.

Serum Alkaline Phosphatase

Alkaline phosphatase in serum was determined by the two-point modification (Kachamar and Moss, 1976) of McComb and Bowers' (1972) continuous monitoring procedure with some additional modifications. Serum (20 μl) was incubated for 30 minutes at 37°C with 0.5 ml of buffered substrate containing 14.0 mM p-nitrophenylphosphate and 0.5

mM $MgCl_2$ in 0.625 M 2-amino-2-methyl-1-propanol (pH 10.25). After 30 minutes, five ml of 0.05 M NaOH were added to each tube. Duplicate tubes (blanks) were prepared with the addition of serum as the final step. Absorption for test and blank tubes was determined at 405 mμ for calculation of ΔA ($A_{test} - A_{blank}$). The absorption differences were compared to a standard curve determined using p-nitrophenol; values ranged from 0.45 to 3.15 μmoles p-nitrophenol. Values were then converted to International Units.

Leucocyte Determination

The number of leucocytes in whole blood was determined by dilution of 0.1 ml of whole blood in 1.1 ml of 0.5% acetic acid containing gentian violet and quantitation in a hemocytometer chamber.

Lectin-dependent Lymphocyte-mediated Cytolysis

Lectin-dependent lymphocyte-mediated cytolysis was determined by the method of Davignon and Laux (1978). Effector cells were prepared from spleens removed from control and DBT-treated rats. These were kept at 4°C in RPMI 1640 complete for no longer than 2 hours prior to assay. A crude preparation of lymphocytes was expressed from spleens with a rubber policeman. Cells were washed and then resuspended in medium containing 0.83% ammonium chloride in 0.01 Tris base to lyse red blood cells. The remaining cells were washed three times and resuspended in medium at a concentration of 5×10^6 cells/ml.

^{51}Cr-target cells were prepared from an intraperitoneal inoculum of EL4 leukemic cells in syngeneic mice (C56BL/6[H-2^{b}]). These cells ($5\text{-}10 \times 10^{6}$) were incubated for 60 minutes at 37°C with 100 µCi of sodium chromate.

Samples were prepared and incubated for 4 hours in a reaction mixture containing 5×10^{5} effector cells, 2×10^{4} target cells, and 1.0 µg of phytohemagglutinin (PHA) or concanavalin A (Con A) in a final volume of 0.3 ml. Spontaneous lysis and 100% lysis were determined by replacing effector cells with medium and distilled water, respectively. After incubation, each mixture was diluted to 2.0 ml with RPMI 1640 and 1.0 ml was counted in a Beckman Gamma 300 Counter. Percent lysis was calculated with the following formula:

$$\text{Percent Lysis} = \frac{\text{Sample Lysis} - \text{Spontaneous Lysis}}{\text{100\% Lysis} - \text{Spontaneous Lysis}}$$

Delayed Hypersensitivity

Rats were tested for delayed-type hypersensitivity to DBT by foot-pad injection (Turk, 1980).

DBT was prepared for foot-pad injection by dissolving in ethanol (100 mg DBT/ml) and adding to 0.25 M sucrose with 0.05 M Tris HCl (pH 7.5) for final concentrations of 5 mg DBT/ml and 5% ethanol. Liver was prepared for injection by homogenizing 1.0 g tissue in 5.0 ml 0.25 M sucrose/0.05 M Tris HCl (pH 7.5), adding ethanol, and diluting to a final concentration of 20 mg liver/ml and 5% ethanol. Liver conjugated

with DBT was prepared by incubating a mixture of DBT (5 mg/ml) and liver (20 mg/ml) in 0.25 M sucrose/0.05 M Tris HCl (pH 7.5) for 1 hour at 4°C.

Binding of DBT to liver was established by dialyzing preparations of liver, DBT, and DBT/liver, similar to those described above, against 0.05 M Tris HCl (pH 7.5) with two changes and quantitating retained DBT by atomic absorption spectrophotometry (Perkin Elmer Model 460 Atomic Absorption Spectrophotometer). DBT/liver samples were also dialyzed in the presence of dimercaprol (12 mg/ml) to assure that the DBT was permeable to the dialysis tubing in spite of its relative insolubility. Samples were prepared for spectrophotometric analysis according to the method of Murthy et al. (1973) by digestion in concentrated nitric acid until clarity, evaporation to near dryness, and dilution in 5% nitric acid. Tin metal dissolved in 10% HCl was used as a standard.

Delayed-type hypersensitivity was determined by challenging control and DBT-treated rats with 0.1 ml vehicle, liver, DBT, or DBT/liver in the right foot-pad 13 days after the start of DBT treatment. Foot-pad swelling was assessed by volume displacement, using a solution of Wright's stain for visibility, and right/left foot volume ratios were calculated.

Immunofluorescence

Direct immunofluorescence (Kawamura, 1977) against rat IgG was performed on control and DBT-treated rat livers using Raine's variation of this standard method (personal communication). Frozen sections were prepared from fresh liver and cut at 4 μ. Tissue was placed in acetone

for 10 minutes, 95% ethanol for 20 minutes, and rinsed twice in phosphate buffered saline. Duplicate slides were incubated with control rat serum for 30 minutes at room temperature after which all slides were incubated for 30 minutes at room temperature with fluorescented rabbit anti-rat IgG (F/P = 4.1). After rinsing twice with phosphate buffered saline, slides were coverslipped with Aqua Mount^{R} (Lerner Labs, Stamford, CT), nonfluorescing medium.

Statistical Methods

Statistical analyses performed included Students' t-test, linear regression, analyses of variance (one-way and multifactorial classifications), and Newman Keuls pairwise comparisons. Acceptable level for a test of significance in these studies was at $P<0.05$. Formulae applicable to these analyses follow:

a) Mean = $\bar{x} = \Sigma x/n$

b) Variation = $\Sigma(x-\bar{x})^2$ = Sum of Squares = SS

c) Variance = $\Sigma(x-x)^2$ = Mean of Squares = MS

d) Standard Deviation = $s = \sqrt{\frac{\Sigma(x-\bar{x})^2}{n-1}}$

e) Standard Error of $\bar{x} = s_{\bar{x}} = s/\sqrt{n}$ = SEM

f) Regression Equation = $\bar{y}_x = a + bx$

g) Correlation Coefficient = $r = \sqrt{r^2} = \sqrt{1 - \frac{\Sigma(y-\bar{y}_x)^2}{\Sigma(y-\bar{y})^2}}$

h) Students' t statistic = $$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{SS_1 + SS_2}{n_1 + n_2 - 2}\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}}$$

i) Studentized Range Statistic = $$q = \frac{\bar{T}_{large} - \bar{T}_{small}}{\sqrt{MS_{error}/n}}$$

j) One-way Analysis of Variance* (ANOVA)

	SS	df	MS	F
Between	$\Sigma T_j^2/n - G^2/kn$	k-1	SS/k-1	MS_{bet}/MS_{WI}
Within	$\Sigma\Sigma x_j^2 - \Sigma T_j^2/n$	k(n-1)	SS/k(n-i)	
Total	$\Sigma\Sigma x_j^2 - G^2/kn$	kn-1	SS/kn-1	

F [k-1,k(n-1)]

i) 2 × 2 Analysis of Variance* (ANOVA)

	SS	df	MS
A	$\Sigma A^2/nq - G^2/npq$	p-1	SS/p-1
B	$\Sigma B^2/np - G^2/npq$	q-1	SS/q-1
AB	$\Sigma (AB)^2/n - \Sigma A^2/nq - \Sigma B^2/np + G^2/npq$	(p-1)(q-1)	SS/(p-1)(q-1)
Error	$\Sigma\Sigma\Sigma x^2 - \Sigma (AB)^2/n$	pq(n-1)	SS/pq(n-1)
Total	$\Sigma\Sigma\Sigma x^2 - G^2/npq$	pqn-1	SS/pqn-1

$F_A = MS_A/MS_E$ F[p-1,pq(n-1)]

$F_B = MS_B/MS_E$ F[q-1,pq(n-1)]

$F_{AB} = MS_{AB}/MS_E$ F[(p-1)(q-1),pq(n-1)]

*The necessary corrections for unequal n were made by Statistical Analysis Systems (SAS), University of Rhode Island.

RESULTS

0 to 4 Day Studies

Since prolyl residues have been shown to be hydroxylated subsequent to incorporation into procollagen (Prockop and Juva, 1965) and such hydroxylation does not occur to a significant extent in noncollagen protein, prolyl hyroxylase activity (PHA) can be used as a measure of collagen synthesis in rat liver. The effect of DBT administration from 0 to 4 days on PHA in rat liver homogenates is shown in Figure 1. There was a gradual increase in activity reaching greater than 220% of control by day 4.

Although PHA has been shown to be a sensitive indicator of collagen synthesis in the liver (Mann *et al.*, 1979), 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 (Diegelmann *et al.*, 1975). A gradual increase in collagen synthesis, reflected by 2,3-^{3}H-proline released from liver protein by collagenase, was observed from 0 to 4 days of DBT administration reaching approximately 500% of control by day 4 (Figure 2). This was not accompanied by an increase in noncollagen synthesis, as measured by incorporation of 2,3-^{3}H-proline into collagen-resistant protein. There was a significant correlation (r=0.94) between the activity of prolyl hydroxylase and *in vitro* collagen synthesis (Figure 3).

Since elevated serum alanine transaminase is commonly associated with hepatic inflammation (DeRitis *et al.*, 1965), the activity of this

Figure 1.

EFFECT OF DIBUTYLTIN[a] (DBT) ON HEPATIC PROLYL HYDROXYLASE ACTIVITY[b] IN RATS AFTER 0 TO 4 DAYS OF TREATMENT.

a) DBT in corn oil was administered at a dose of 20 mg/kg/day, p.o.; controls received equivalen amounts of corn oil.

b) Enzyme activity in liver homogenates was quantitated by tritium release assay from labelled collagen substrate as described in the methods. Seven experimental animals were used per day. Values are mean $\pm$ S.E.M. Analysis of variance, one-way classification for the treatment variable day, significant at $P<0.05$.

c) Significant at $P<0.05$ vs. control (Newman Keuls pairwise comparisons).

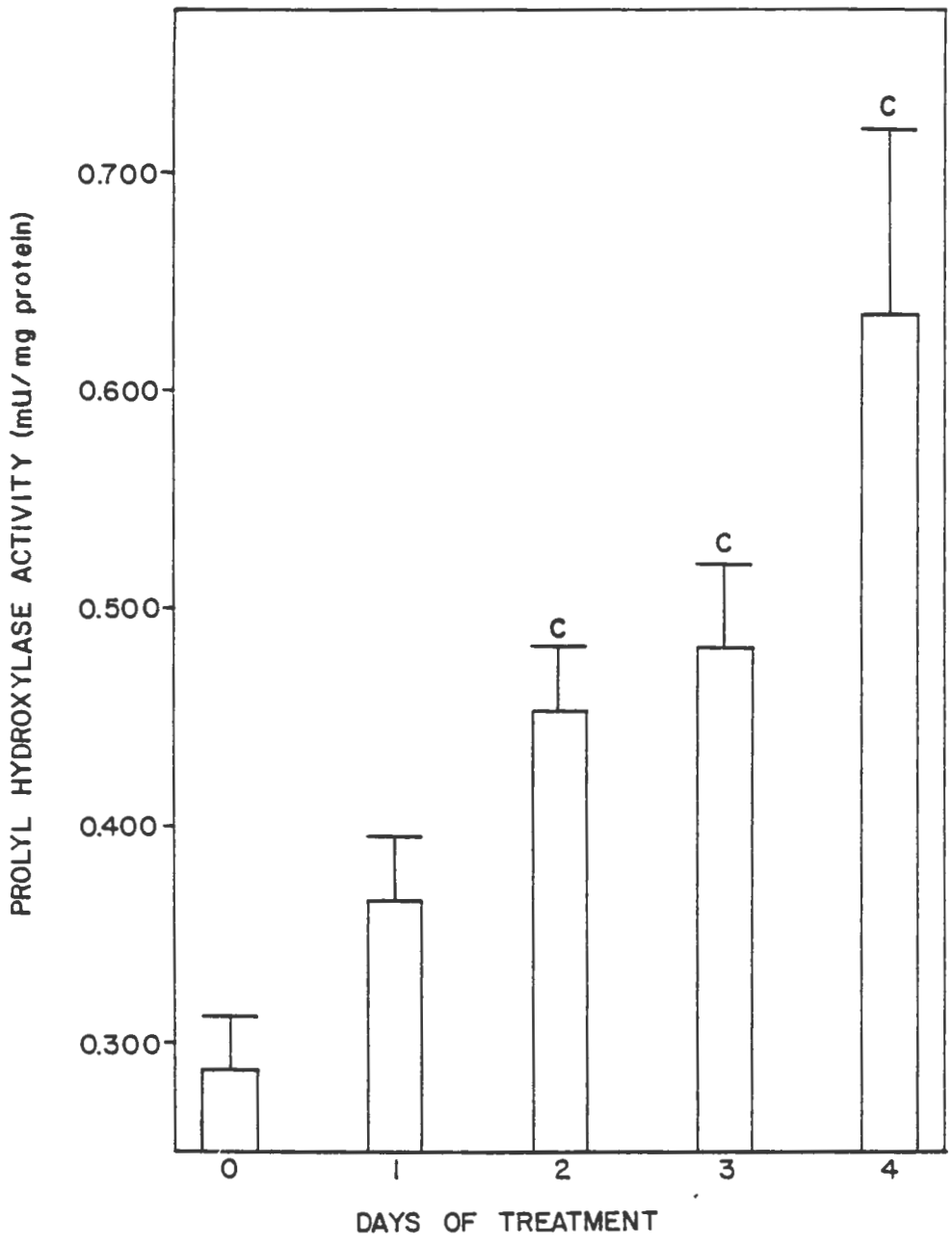

PROLYL HYDROXYLASE ACTIVITY (mU/mg protein)
0.700
0.600
0.500
0.400
0.300
C
C
C
0
1
2
3
4
DAYS OF TREATMENT

Figure 2.

EFFECT OF DIBUTYLTIN[a] (DBT) ON IN VITRO COLLAGEN AND NONCOLLAGEN PROTEIN SYNTHESIS[b] IN RAT LIVER AFTER 0 TO 4 DAYS OF TREATMENT.

a) DBT in corn oil was administered at a dose of 20 mg/kg/day, p.o.; controls received equivalent amounts of corn oil.

b) Collagenase digestion was performed on homogenates of liver minces following 24 hour incubation with 2,3-^{3}H-proline as described in the methods. Collagen is represented by label in supernate as a result of collagenase digestion. Noncollagen protein is represented by labelled collagenase-resistant protein in the pellet. Three experimental animals were used per day except for control where two were used. Values are mean $\pm$ S.E.M. Analysis of variance, one-way classification for the treatment variable day, significant at $P<0.05$ for the response variable collagen.

c) Significant at $P<0.05$ vs. control and day 1 (Newman Keuls pairwise comparisons).

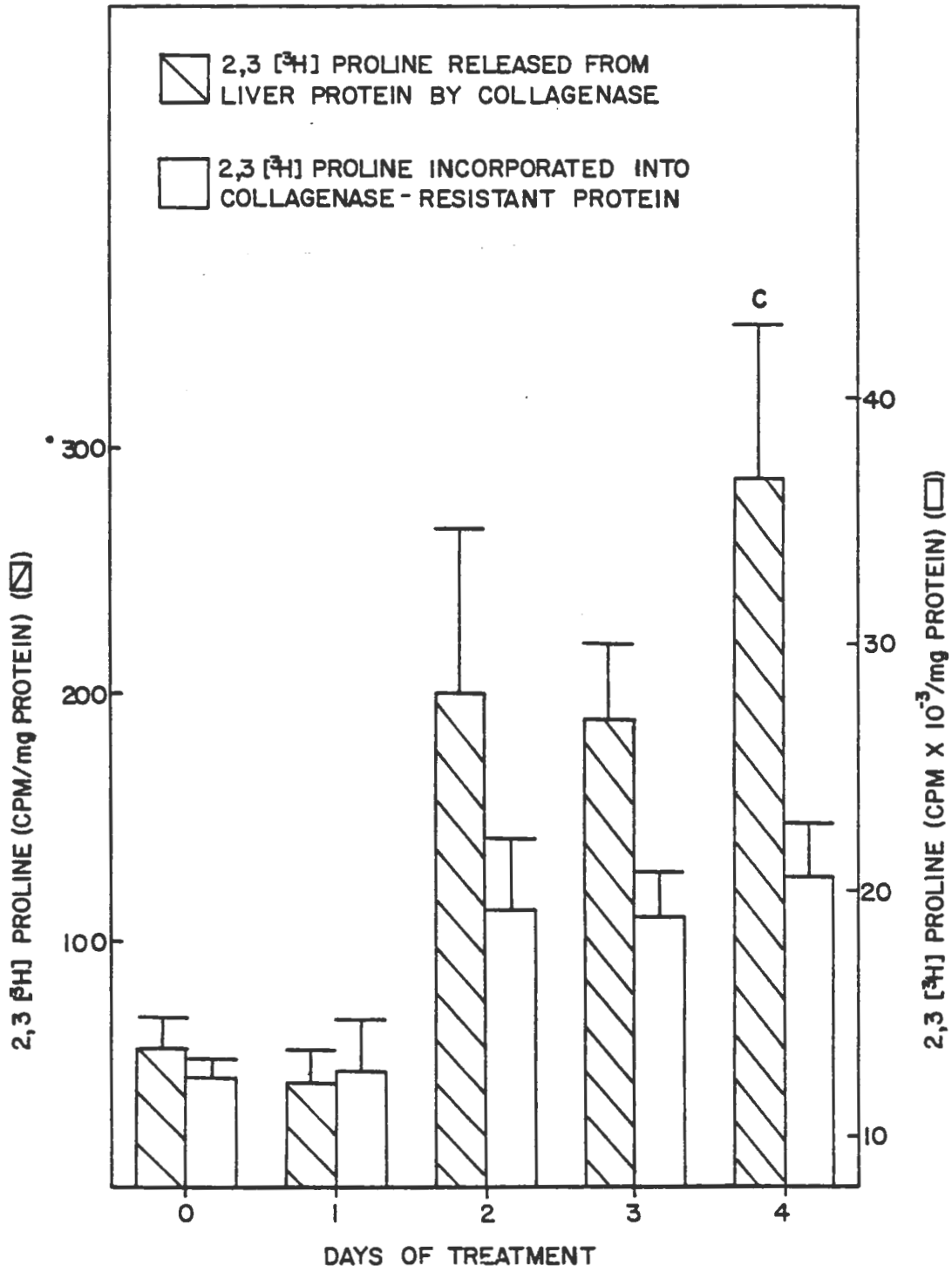
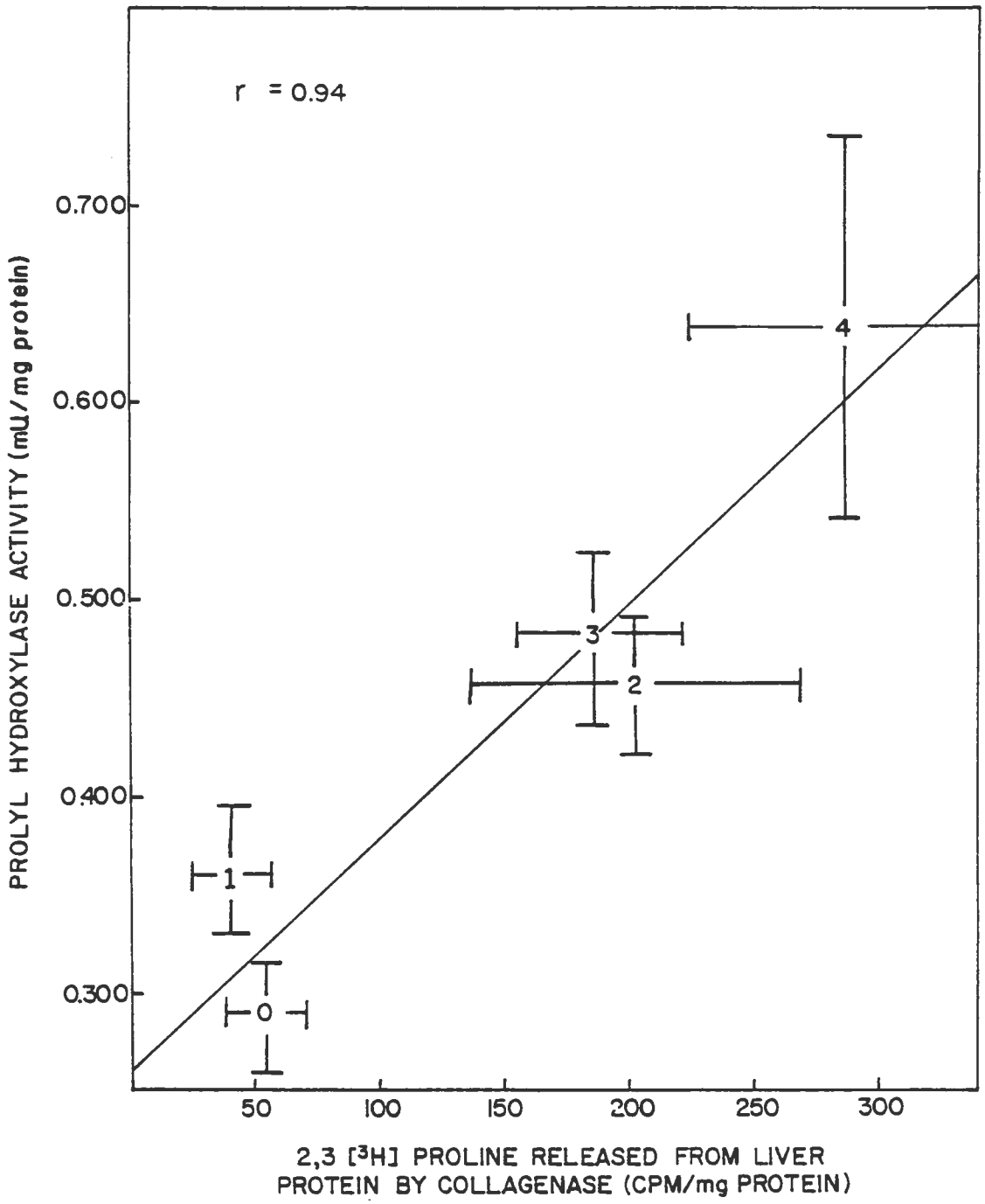

2,3 [³H] PROLINE RELEASED FROM LIVER PROTEIN BY COLLAGENASE
2,3 [³H] PROLINE INCORPORATED INTO COLLAGENASE-RESISTANT PROTEIN
C
300
200
100
40
30
20
10
0
1
2
3
4
DAYS OF TREATMENT
2,3 [³H] PROLINE (CPM/mg PROTEIN) (▨)
2,3 [³H] PROLINE (CPM X 10⁻³/mg PROTEIN) (□)

Figure 3.

CORRELATION BETWEEN HEPATIC PROLYL HYDROXYLASE ACTIVITY AND IN VITRO COLLAGEN SYNTHESIS[a].

a) Each point represents mean ± S.E.M. of prolyl hydroxylase activity and in vitro collagen synthesis on the specified day of treatment. Experimental details are described in Figures 1. and 2. and the methods.


r = 0.94
0.700
0.600
0.500
0.400
0.300
PROLYL HYDROXYLASE ACTIVITY (mU/mg protein)
50
100
150
200
250
300
2,3 [3H] PROLINE RELEASED FROM LIVER PROTEIN BY COLLAGENASE (CPM/mg PROTEIN)
0
1
2
3
4

enzyme was determined in serum from rats after 4 days of DBT administration. An increase in serum alanine transaminase of approximately 400% of control was observed at this time (Table 1).

Histological observations of livers from rats treated with DBT for 0 to 4 days revealed increasing frequency of the appearance of periportal inflammation and bile duct proliferation during this period (Figures 5 and 6) as compared to control (Figure 4). The infiltration of predominantly polymorphonuclear cells was extensive in the proximity of ductular cells.

12-Day Studies

The effect of DBT on parameters of hepatic collagen metabolism after 12 days of treatment is shown in Table 2. These results have been described previously (Yermakoff, 1978). The activity of prolyl hydroxylase was increased to nearly 150% of control in DBT-treated rats. There was no significant change in either the amount of label released from liver protein by collagenase or the amount incorporated into collagenase-resistant protein. However, the percent of total labelled protein digested by collagenase was increased approximately two-fold in animals treated with DBT, reflecting a change in these two parameters relative to each other. Finally, DBT produced a greater than two-fold increase in hepatic hydroxyproline content, representing an increase in collagen accumulation.

Since certain serum enzymes have been shown to be useful indicators of both the degree and type of liver injury (DeRitis et al., 1965),

Table 1. Effect of dibutyltin dichloride[a] (DBT) on serum alanine transaminase[b] (ALTA) after 4 days of treatment.

Group	No. of Rats	ALTA (mIU/0.2ml)
Control	6	6.18 ± 0.32
DBT	5	23.00 ± 3.44[c]

[a]DBT was administered at a dose of 20 mg/kg/day, p.o.; controls received equivalent amounts of corn oil.

[b]Serum ALTA was determined colorimetrically as described in the methods. Values are mean ± S.E.M.

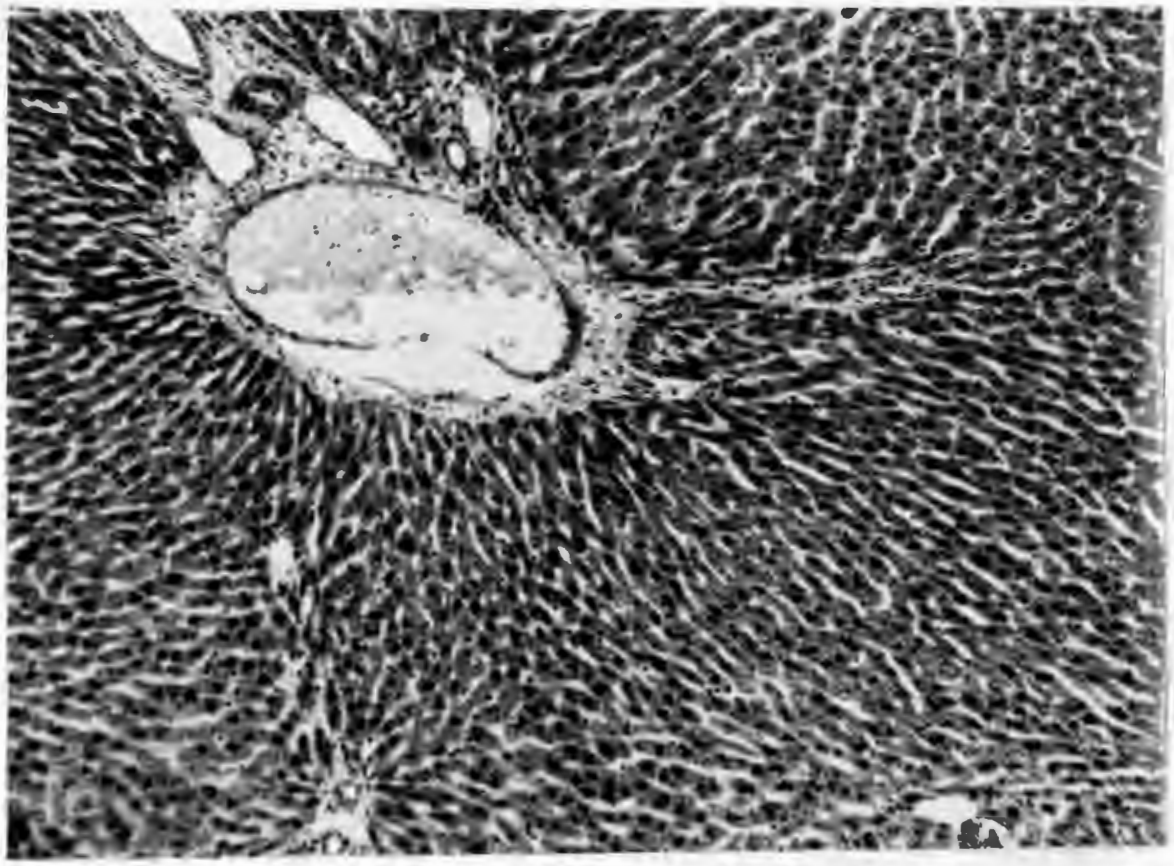
[c]Significant at $P < 0.05$ vs. control (two-tailed Student's t-test).

Figure 4.

PHOTOMICROGRAPH OF LIVER FROM A CONTROL RAT SHOWING A CENTRAL VEIN AND PORTAL TRIAD. TRICHROME STAIN x 100.

Figure 5.

RAT LIVERS AFTER 1 DAY (A) AND 2 DAYS (B) OF DBT ADMINISTRATION (20 mg/kg/day, p.o.). PERIPORTAL INFLAMMATION AND DISORGANIZATION OF DUCTULAR CELLS ARE APPARENT IN (B). TRICHROME STAIN x 100.



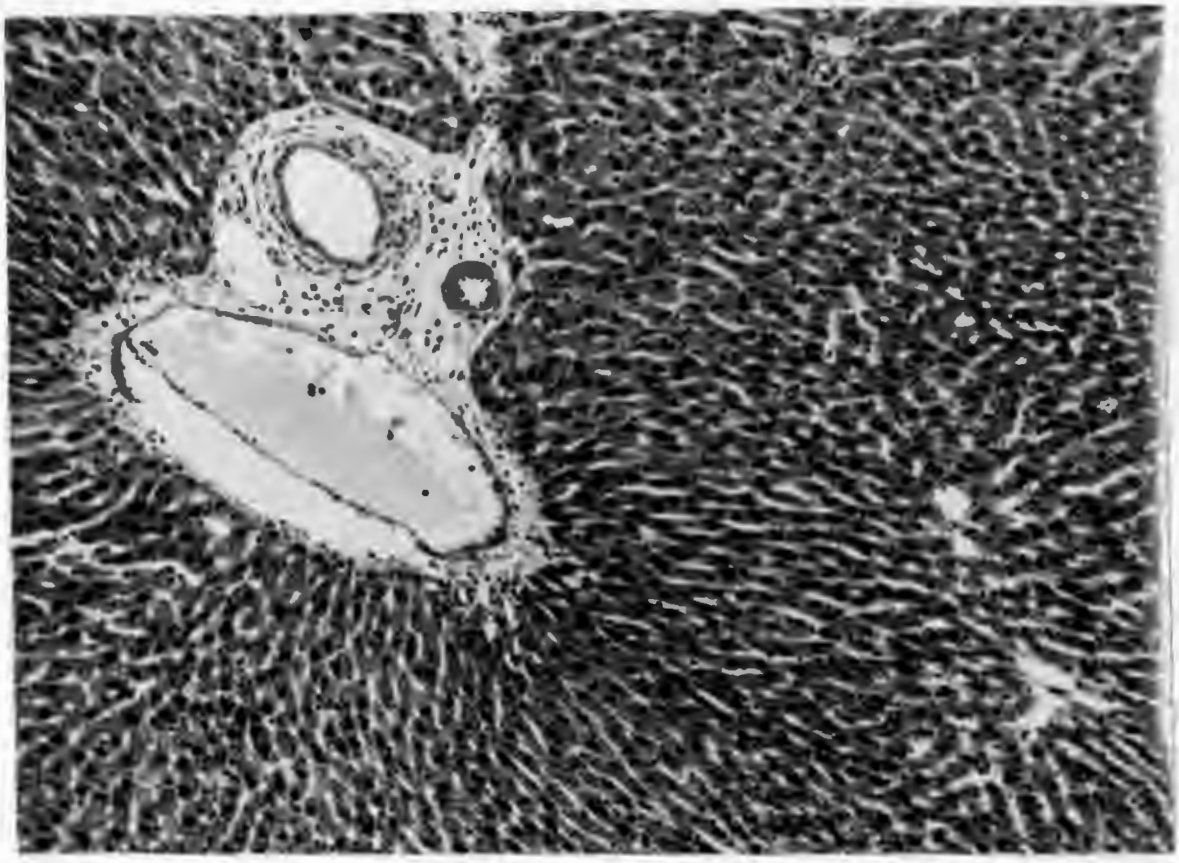


Figure 6.

PHOTOMICROGRAPH OF RAT LIVERS AFTER 3 DAYS (A) AND 4 DAYS (B) OF DBT ADMINISTRATION (20 mg/kg/day, p.o.). DISORGANIZED DUCTULAR CELL PROLIFERATION AND PERIPORTAL INFLAMMATION ARE SEEN IN (A) AND, MORE PERVASIVELY, IN (B). TRICHROME STAIN x 100.

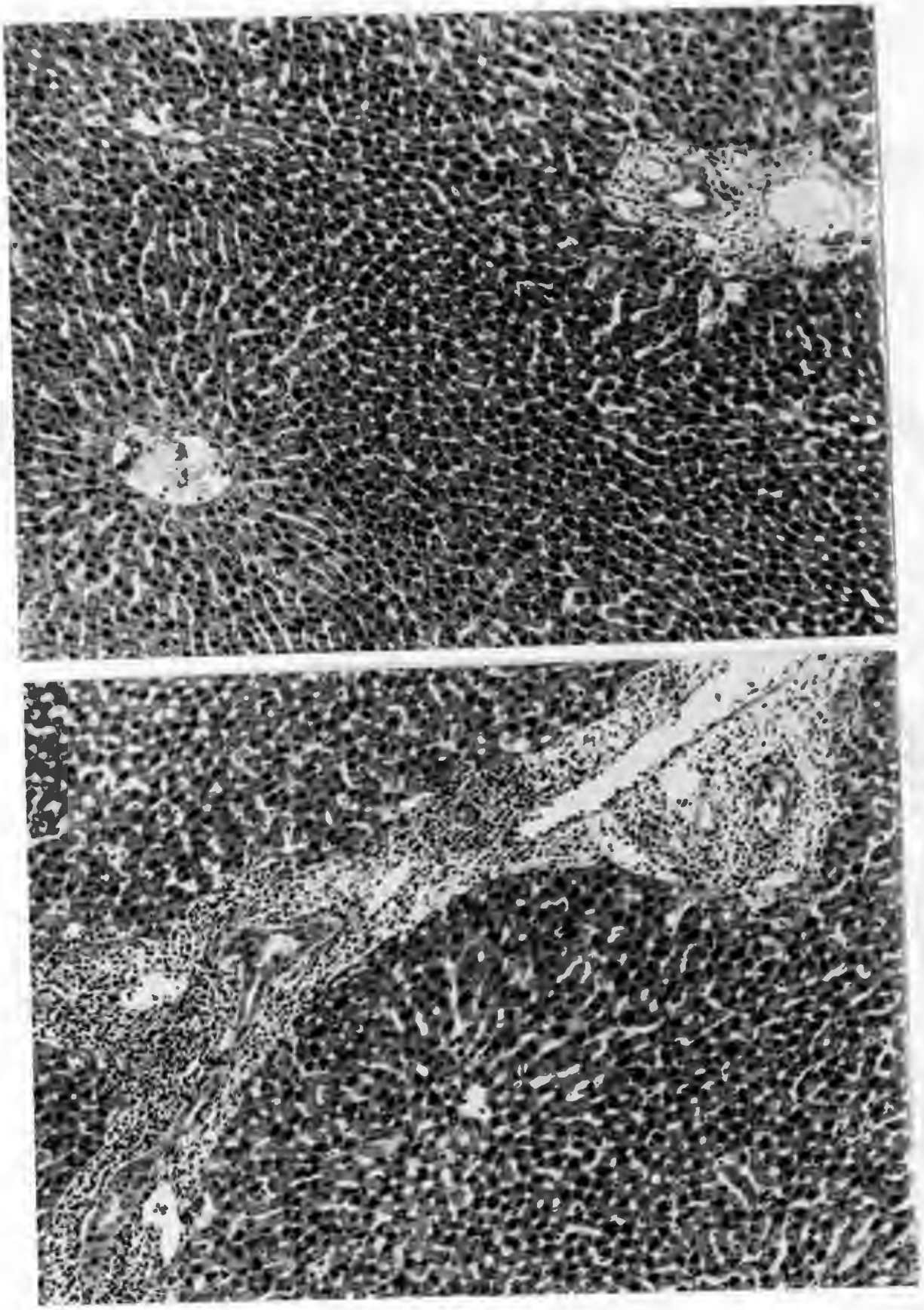


Table 2. Effect of dibutyltin dichloride[a] (DBT) on parameters[b] of hepatic collagen metabolism after 12 days of treatment.

	Control	DBT
Prolyl Hydroxylase Activity (mU/mg protein)	0.282 ± 0.010 (10)	0.412 ± 0.029[c] (12)
In Vitro Collagen Synthesis		
4-^{3}H-proline released from liver protein by collagenase (cpm/mg protein)	307 ± 81 (6)	440 ± 75 (5)
4-^{3}H-proline incorporated into collagenase resistant protein (cpm x 10^{-3}/mg protein)	19.2 ± 3.0 (6)	13.4 ± 0.9 (5)
% total labelled protein digested by collagenase	0.30 ± 0.08 (6)	0.59 ± 0.08[c] (5)
Hydroxyproline Content (ug/g liver wet weight)	125 ± 4 (6)	266 ± 4[c] (6)

[a]DBT was administered in corn oil 20 mg/kg, q.o.d., p.o.; controls received equivalent amounts of corn oil.

[b]Data and methods have been described previously (Yermakoff, 1978). Values are mean ± S.E.M.

[c]Significant vs. control at $P < 0.05$ (two-tailed Student's t-test).

alanine transaminase and alkaline phosphatase activities were determined in serum from DBT-treated rats after 12 days of administration. There was no significant elevation in the activity of either of these two enzymes in treated animals (Table 3).

Histological changes in these rats included extensive infiltration of both polymorphonuclear and mononuclear cells, proliferation and injury to ductular cells, and fibrosis within the portal tracts (Figures 7 and 8).

The effect of the nonsteroidal antiinflammatory drug, naproxen, on the activities of prolyl hydroxylase and alanine transaminase when administered alone and in combination with DBT for 12 days is shown in Figure 9. DBT produced an increase in PHA which was 146% of control, while naproxen had no significant effect on the activity of this enzyme. Naproxen and DBT administered together produced an increase in PHA that was 127% of control. The interaction between these two compounds was not significant as measured by multifactorial analysis of variance with the treatment variables DBT and naproxen. However, there was no significant difference between PHA in the naproxen and DBT/naproxen groups. The activity of serum alanine transaminase was not elevated in any group.

Age-related Studies

Since DBT has been shown to produce thymotoxicity and impaired cell-mediated immunity in rats treated with DBT pre- and postnatally (Seinen et al., 1977b) and immunologic processes were suspected in

Table 3. Serum enzymes after 12 days of dibutyltin dichloride[a] (DBT) administration.

Group	Alanine Transaminase[b] Activity (mIU/0.2ml)	Alkaline Phosphatase[b] Activity (mIU/ml)
Control	4.98 ± 0.25 (7)	266 ± 19 (8)
DBT	6.26 ± 1.05 (9)	268 ± 27 (10)

[a]DBT was administered at a dose of 20 mg/kg, q.o.d., p.o.; controls received equivalent amounts of corn oil.

[b]Enzyme activity was determined colorimetrically as described in the methods. Values are mean ± S.E.M.

Figure 7.

A PORTAL TRACT AFTER 12 DAYS OF DBT ADMINISTRATION (20 mg/kg/day, p.o.). FIBROUS TISSUE SURROUNDS THE PORTAL VEIN. PERIPORTAL INFLAMMATION IS EXTENSIVE AND DISORGANIZED DUCTULAR CELL PROLIFERATION IS APPARENT. TRICHROME STAIN x 100.

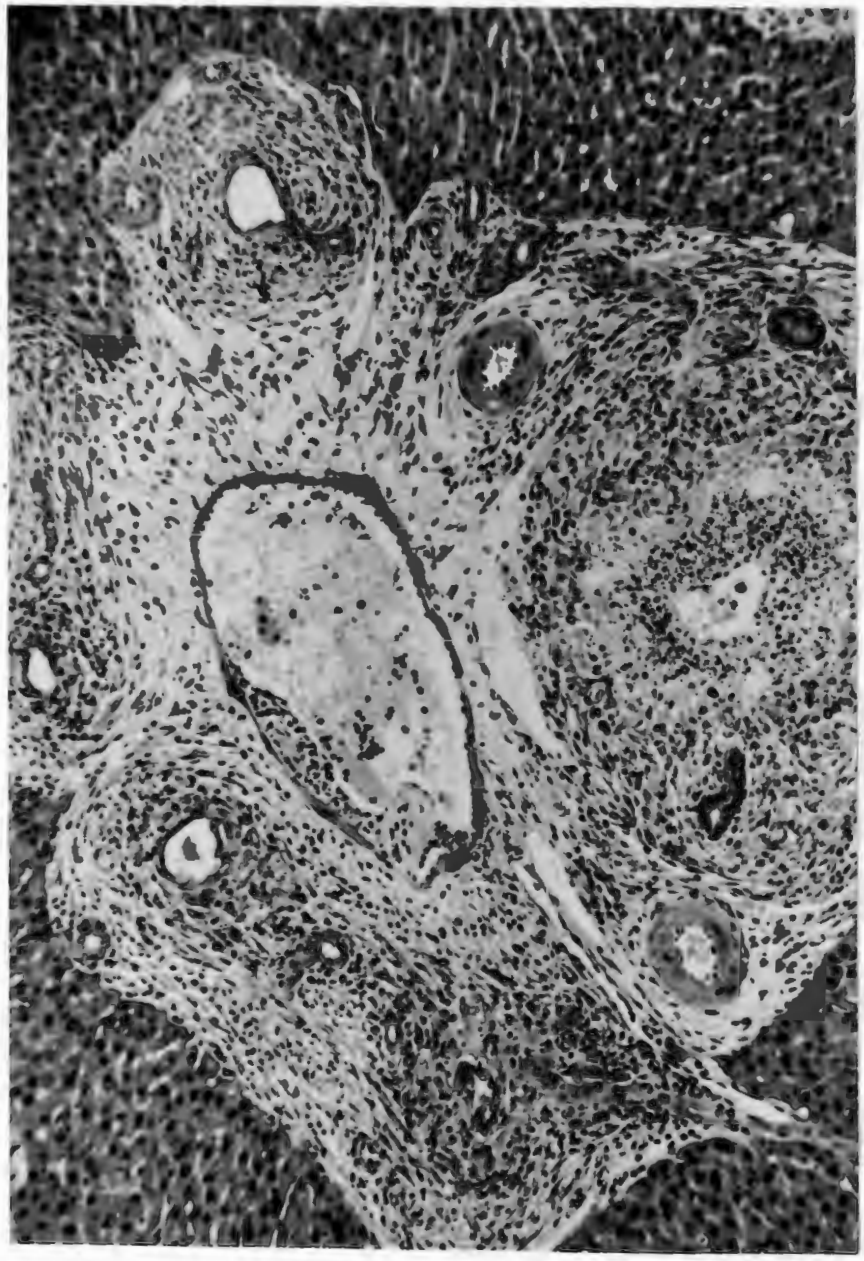


Figure 8.

PORTION OF A PORTAL TRACT AFTER 12 DAYS OF DBT ADMINISTRATION (20 mg/kg, q.o.d., p.o.). INFLAMMATORY CELLS SURROUND INJURED AND PROLIFERATING DUCTULAR CELLS. TRICHROME STAIN x 100.

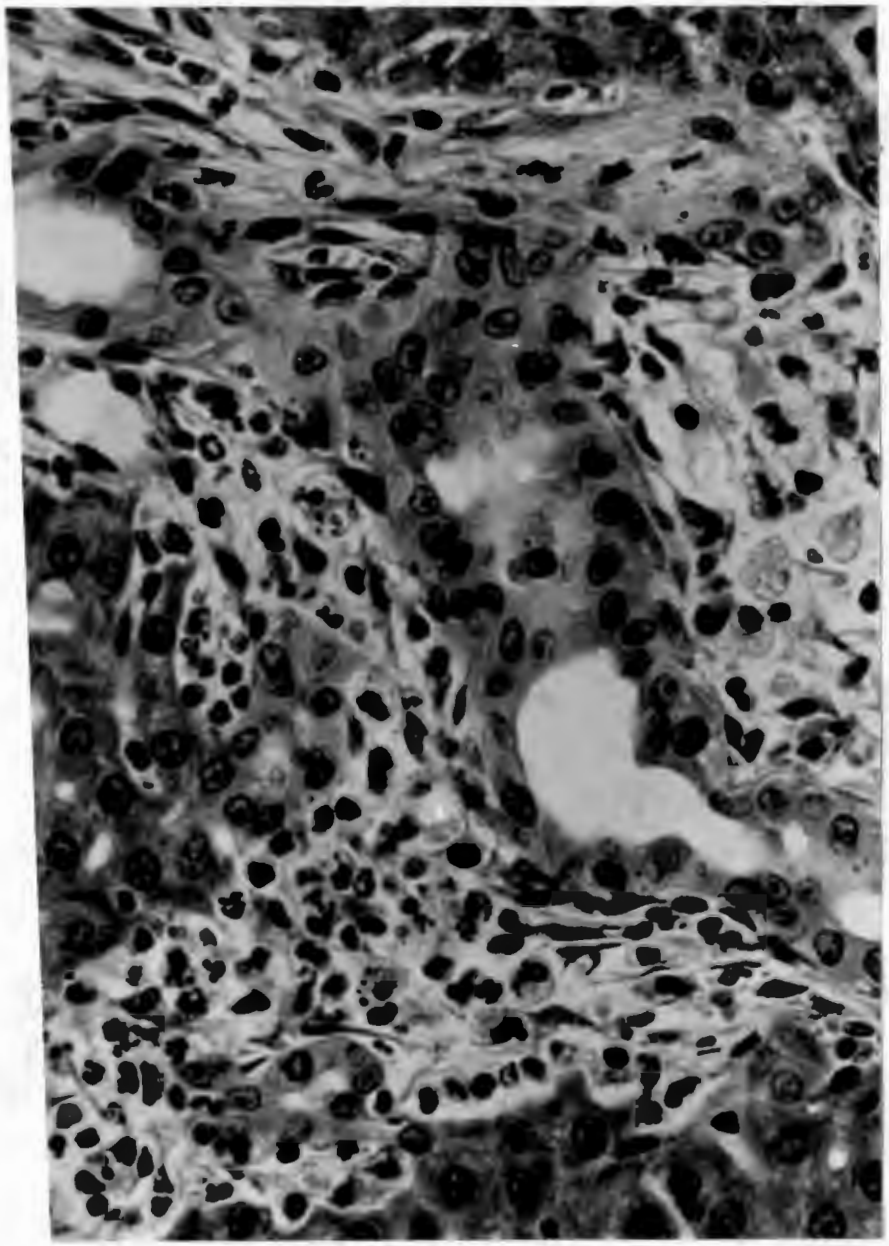


Figure 9.

EFFECTS OF DIBUTYLTIN[a] (DBT) AND NAPROXEN[a] (NAP) SEPARATE AND COMBINED ON HEPATIC PROLYL HYDROXYLASE ACTIVITY[b] AND SERUM ALANINE TRANSAMINASE ACTIVITY[c] IN RATS AFTER 12 DAYS OF TREATMENT.

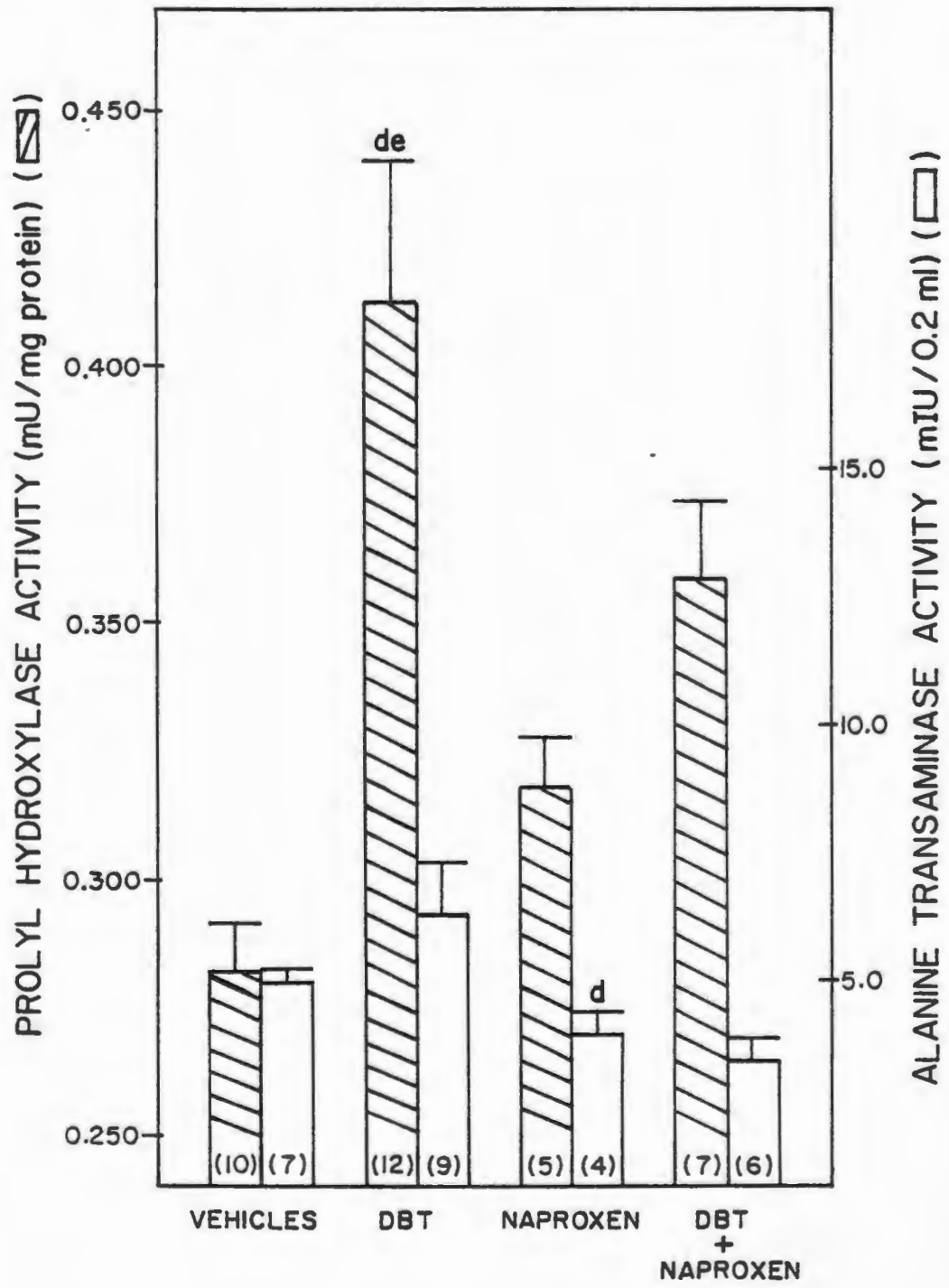
a) DBT and NAP were administered in corn oil at doses of 20 mg/kg, q.o.d., p.o., and 15 mg/kg, b.i.d., p.o., respectively; controls received equivalent amounts of corn oil.

b) Prolyl hyroxylase activity (PHA) was determined in liver homogenates by ^{3}H-release assay from labelled collagen substrate as described in the methods.

c) Serum alanine transaminase activity (ALTA) was determined colorimetrically as described in the methods. All values are mean $\pm$ S.E.M.

d) Analysis of variance, multifactorial classification for the treatment variables DBT and NAP and the response variable PHA or ALTA, significant effect at $P<0.05$.

e) Analysis of variance, one-way classification for the treatment variable DBT and the response variable PHA or ALTA, significant at $P<0.05$.


PROLYL HYDROXYLASE ACTIVITY (mU/mg protein) (▨)
ALANINE TRANSAMINASE ACTIVITY (mIU/0.2 ml) (□)
0.450
0.400
0.350
0.300
0.250
15.0
10.0
5.0
de
d
(10)
(7)
(12)
(9)
(5)
(4)
(7)
(6)
VEHICLES
DBT
NAPROXEN
DBT + NAPROXEN

DBT-induced cholangitis, a possible inverse relationship between these two phenomena was investigated in age-related studies. The effect of DBT on hepatic PHA after 12 days of treatment invarious age groups is shown in Table 4. Enzyme activity was 125%, 146%, and 215% of control in rats 4, 9, and 14 weeks of age at the start of treatment. Quantitation of peripheral blood leucocytes, presented in Table 5, shows a DBT-induced elevation in these cells only in the rats 9 and 14 weeks of age at the start of treatment. The effects of DBT on rat body weight in these three age groups is compared in Figure 10; there was a significant difference between control and DBT-treated animals in the 9 and 14 week groups only.

Hepatic histopathology in rats 14 weeks of age at the start of treatment was similar to that observed in the 9-week old group, which was described above. However, the 4-week old group showed little evidence of the usual histopathalogic lesions induced by DBT. Periportal inflammation and bile duct proliferation were moderate or absent in these livers (Figure 11).

Immunology

Since DBT has been shown to produce thymotoxicity in weanling rats following both intravenous administration or incorporation of DBT in the diet (Seinen *et al.*, 1977b), the effect of DBT on thymus weight in 4-week old rats in the dosage regimen described herein was investigated. Table 6 shows a striking decrease in thymus weight to less than one

Table 4. Effect of dibutyltin dichloride[a](DBT) on prolyl hydroxylase activity[b](PHA) after 12 days of treatment in rats 4,9, and 14 weeks of age at the start of treatment.

Group	No. of Rats	PHA (mU/mg protein)	% Control
		4 WEEKS	
Control	6	0.307 ± 0.018	100 ± 6
DBT	10	0.386 ± 0.014[c]	125 ± 5[c]
		9 WEEKS	
Control	10	0.282 ± 0.010	100 ± 4
DBT	12	0.412 ± 0.029[c]	146 ± 10[c]
		14 WEEKS	
Control	4	0.136 ± 0.016	100 ± 12
DBT	8	0.292 ± 0.044[c]	215 ± 32[c]

[a]DBT was administered at a dose of 20 mg/kg, q.o.d., p.o.; controls received equivalent amounts of corn oil.

[b]PHA was determined in liver homogenates by the ^{3}H-release assay from labelled collagen substrate as described in the methods. Values are mean ± S.E.M.

[c]Significant at $P < 0.05$ vs. control (one-tailed Student's t-test).

Table 5. Effect of dibutyltin dichloride[a](DBT) on leucocyte count[b] after 12 days of treatment in rats 4, 9, and 14 weeks of age at the start of treatment.

Group	No. of Rats	Leucocytes/cu min	% Control
		4 WEEKS	
Control	5	6,450 ± 507	100 ± 8
DBT	9	5,183 ± 793	80 ± 12
		9 WEEKS	
Control	5	12,130 ± 1,142	100 ± 9
DBT	5	18,940 ± 2,379	156 ± 19
		14 WEEKS	
Control	4	9,700 ± 1,236	100 ± 13
DBT	7	12,114 ± 709	125 ± 7

[a]DBT was administered at a dose of 20 mg/kg, q.o.d., p.o.; controls received equivalent amounts of corn oil.

[b]Leucocytes were quantitated as described in the methods. Values are mean ± S.E.M.

[c]Significant at $P<0.05$ vs. control (one-tailed Student's t-test).

Figure 10.

EFFECTS OF DIBUTYLTIN[a] (DBT) ON RAT BODY WEIGHT[b] AFTER 12 DAYS OF TREATMENT.

a) DBT in corn oil was administered at a dose of 20 mg/kg, q.o.d., p.o.; controls received equivalent amounts of corn oil.

b) The number of experimental animals in each group are shown in parentheses. Values are mean $\pm$ S.E.M. of weight change during DBT treatment.

c) Significant at $P<0.05$ vs. control (one-tailed Students' t-test).

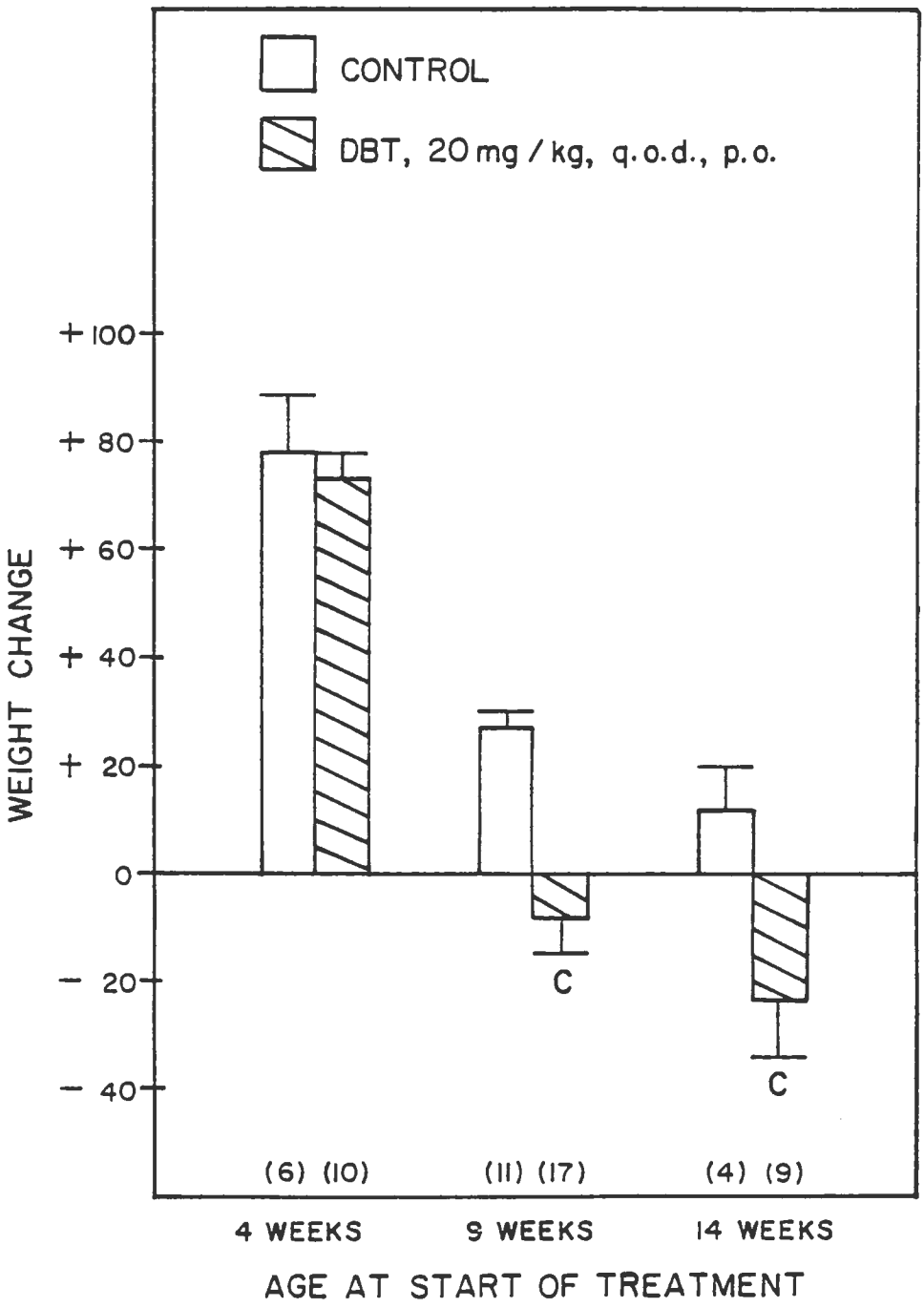

CONTROL
DBT, 20 mg / kg, q.o.d., p.o.
WEIGHT CHANGE
+100
+80
+60
+40
+20
0
−20
−40
C
C
(6) (10)
(11) (17)
(4) (9)
4 WEEKS
9 WEEKS
14 WEEKS
AGE AT START OF TREATMENT

Figure 11.

LIVER FROM 4-WEEK OLD RAT AFTER 12 DAYS OF DBT ADMINISTRATION (20 mg/kg, q.o.d., p.o). TRICHROME STAIN x 100.

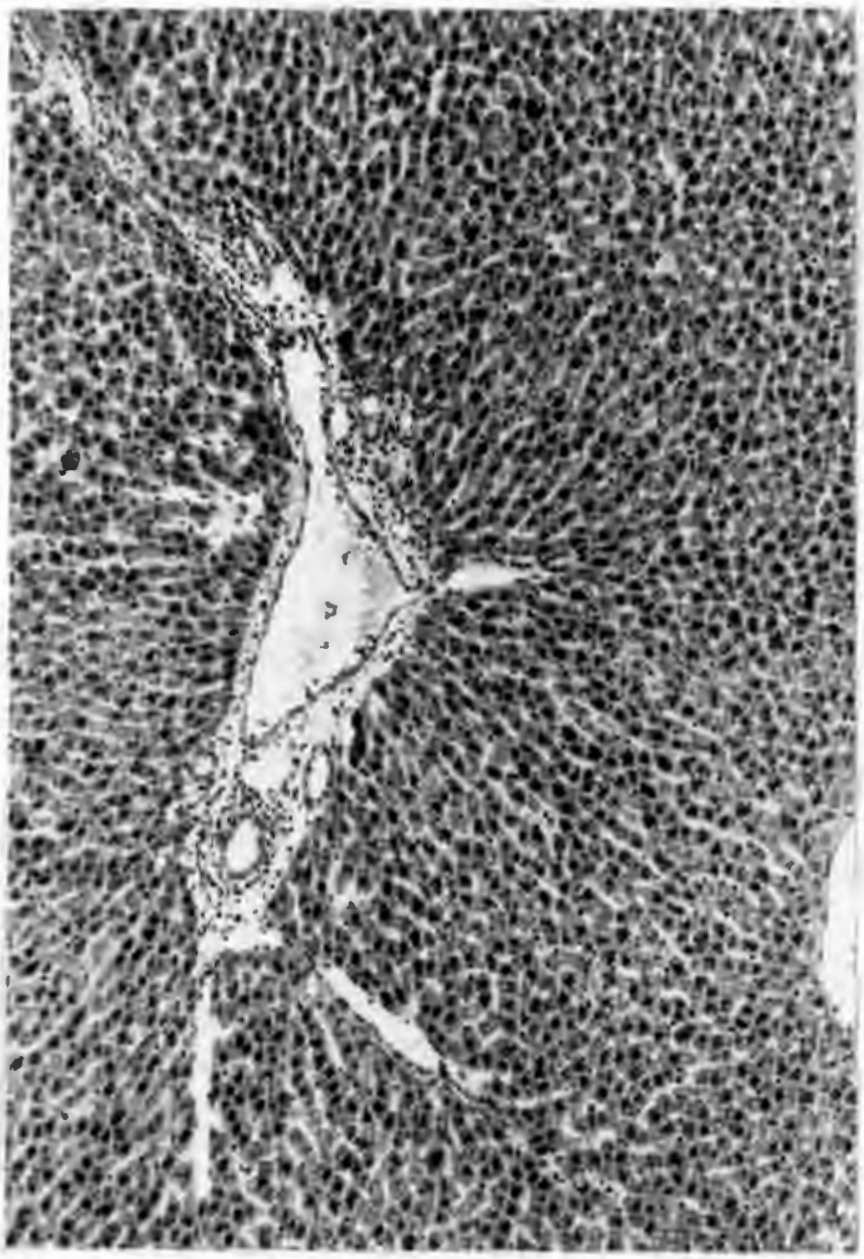


Table 6. Effect of DBT[a] on thymus weight in rats 4 weeks of age.

	No. of Rats	Thymus (g dry weight)	Thymus (g wet weight)	Body Weight (g)	Thymus (mg/g body weight)
Control	6	0.15 ± 0.02	0.82 ± 0.11	182 ± 14	4.4 ± 0.4
DBT	10	0.04 ± 0.01[b]	0.25 ± 0.04[b]	191 ± 6	1.3 ± 0.2[b]

[a]DBT in corn oil was administered 20 mg/kg, q.o.d., p.o. for 12 days; controls received equivalent amounts of corn oil. Values are mean ± S.E.M.

[b]$P<0.05$ vs. control (two-tailed Student's t-test).

third of control when measured by wet or dry organ weight. When normalized for body weight, the decrease in thymus weight is as great.

Immunologic studies of 9-week old rats included determination of lectin-dependent lymphocyte-mediated cytolysis (Davignon and Laux, 1978). The assay is a measure of cytotoxic t-cell activity, in which the presence of lectin precludes the requirement for specific target cells. Lysis of ^{51}Cr-labelled target cells by cytotoxic t-cells was not apparent in either control or DBT treated rats after 12 days of treatment. Percent lysis was approximately 0-2% in both groups.

A second parameter of cell-mediated immunity, delayed-type hypersensitivity, was investigated in rats after 12 days of DBT administration. Challenge doses of DBT were given with this organotin alone or conjugated to a liver macromolecule. Equilibrium analysis studies confirmed that most of the DBT (94%) in this DBT/liver conjugate is non-dialyzable after incubation with liver homogenate as compared to 48% and 42% without liver and in the presence of dimercaprol, respectively. After a challenge dose of DBT or DBT/liver conjugate, both controls and DBT-treated rats showed foot-swelling characteristic of a delayed-type hypersensitivity response (Table 7). However, the equivalent responses in control and treated animals suggested that these results were not due to an immunologic response. Histopathologic observation revealed predominately polymorphonuclear infiltration and extensive tissue injury suggesting acute inflammation (Figure 12).

Investigation of antibodies directed against hepatic antigens and/or DBT in liver from DBT-treated rats was performed by direct immunofluorescence using rabbit anti-rat IgG. Immunofluorescence was localized to,

Table 7. Effect of a challenge dose[a] of dibutyltin (DBT) on rats after 12 days of DBT administration[b].

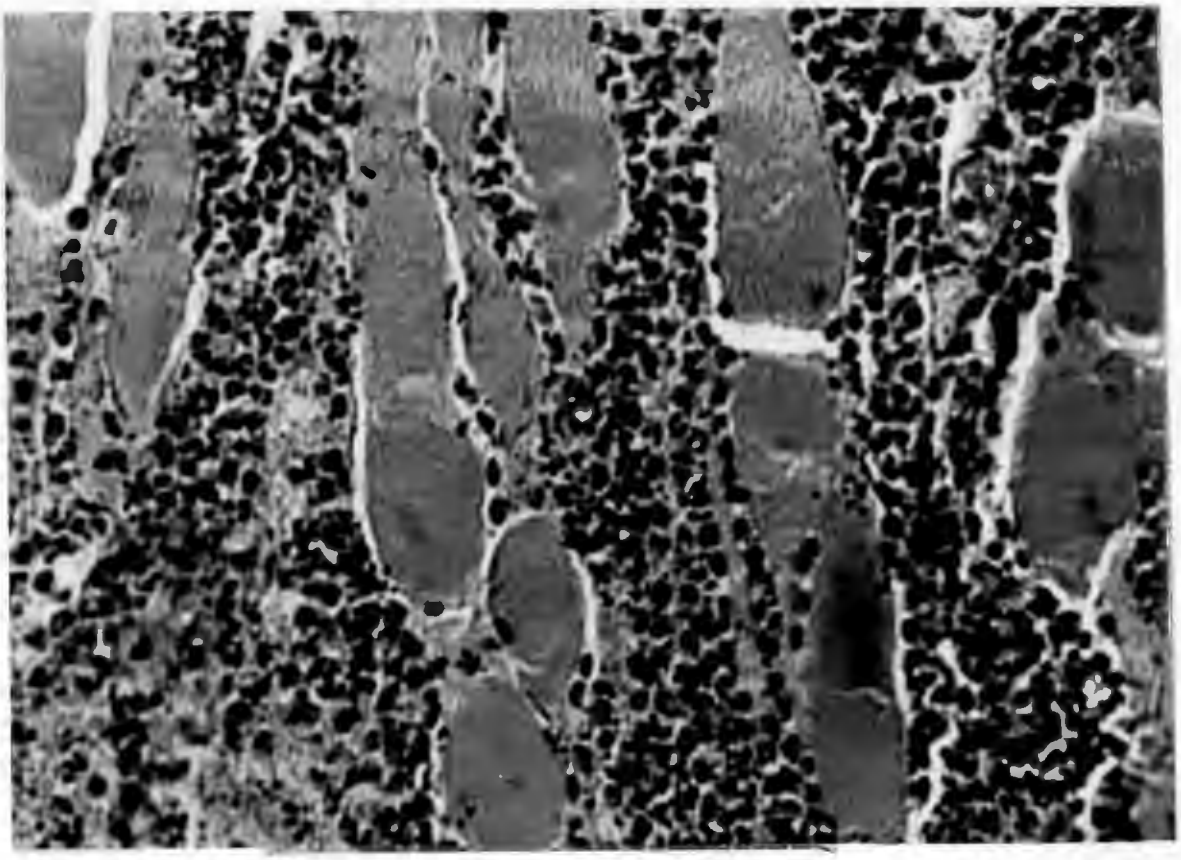
Challenge	Ratio Right/Left Foot Volume	
	CONTROL	DBT
Vehicle	1.0	1.0
	1.0	1.0
	1.0	1.0
Liver	1.0	1.0
(2 mg)	1.0	1.0
	1.0	1.0
DBT	1.3	1.3
(0.5 mg)	1.3	1.5
	1.3	2.3
DBT/Liver	1.8	1.8
(0.5 mg/2 mg)	1.8	1.7
	1.8	1.3

[a] Challenge dose contained the concentrations of DBT and liver indicated above in a volume of 0.1 ml; it was injected in the right foot pad using a 1/4 cc glass syringe and 25 gauge needle.

[b] DBT was administered at a dose of 20 mg/kg, q.o.d., p.o.; controls received equivalent amounts of corn oil.

Figure 12.

MUSCLE FIBERS IN A NORMAL RAT FOOT (A) AND IN A FOOT 24 HOURS AFTER INJECTION OF 0.5 mg DBT INTO THE FOOT PAD (B). ACUTE INFLAMMATION AND DISORGANIZATION OF MUSCLE FIBERS ARE APPARENT IN (B). HEMATOXYLIN-EOSIN STAIN x 400.



but equally intense throughout, the portal tracts. It was suggestive of a reaction against serum IgG rather than antibodies directed against a particular hepatic cell type.

Studies with SKF 525A

The possible relationship between differences in hepatic microsomal metabolism and age-related effects of DBT was investigated using an inhibitor of microsomal enzymes, SKF 525A. SKF 525A and DBT were administered alone and concomitantly to rats for 4 days. The effects of these treatments on PHA and serum alanine transaminase activity are shown in Figure 13. PHA was increased in the DBT group and SKF group to approximately 220% and 250% of control, respectively. The activity of prolyl hydroxylase in the DBT/SKF 525A group was nearly 270% of control and substantially less than what would be anticipated if their effects were additive; as determined by a multifactorial analysis of variance with the treatment variables DBT and SKF 525A, this represented a significant interactive effect between these two compounds. There was no difference in the protein content of liver tissue in all four groups when measured as mg protein/mg liver wet weight. Therefore, prolyl hydroxylase values in the livers of these rats were not a function of altered protein content. The increase in alanine transaminase activity measured in the serum from DBT-treated rats at 4 days time was completely inhibited by SKF 525A.

Observation of livers from SKF 525A-treated rats revealed relatively normal histology (Figure 14). The only obvious pathology was the

Figure 13.

EFFECTS OF DIBUTYLTIN[a] (DBT) AND SKF 525A[a] (SKF) SEPARATE AND COMBINED ON HEPATIC PROLYL HYDROXYLASE ACTIVITY[b] AND SERUM ALANINE TRANSAMINASE ACTIVITY[c] IN RATS AFTER 4 DAYS OF TREATMENT.

a) DBT in corn oil was administered at a dose of 20 mg/kg/day, p.o. and SKF in saline at a dose of 50 mg/kg, b.i.d., i.p.; controls received equivalent amounts of vehicles.

b) Prolyl hydroxylase activity (PHA) was determined in liver homogenates by ^{3}H-release assay from labelled collagen substrate as described in the methods.

c) Serum alanine transaminase activity (ALTA) was determined colorimetrically as described in the methods. Values are mean $\pm$ S.E.M.

d) Analysis of variance, multifactorial classification for the treatment variables DBT and SKF and the response variable PHA and ALTA, significant effect at $P<0.05$ or

e) significant interaction at $P<0.05$.

f) Analysis of variance, one-way classification for the treatment variable DBT and the response variable PHA or ALTA, significant at $P<0.05$.

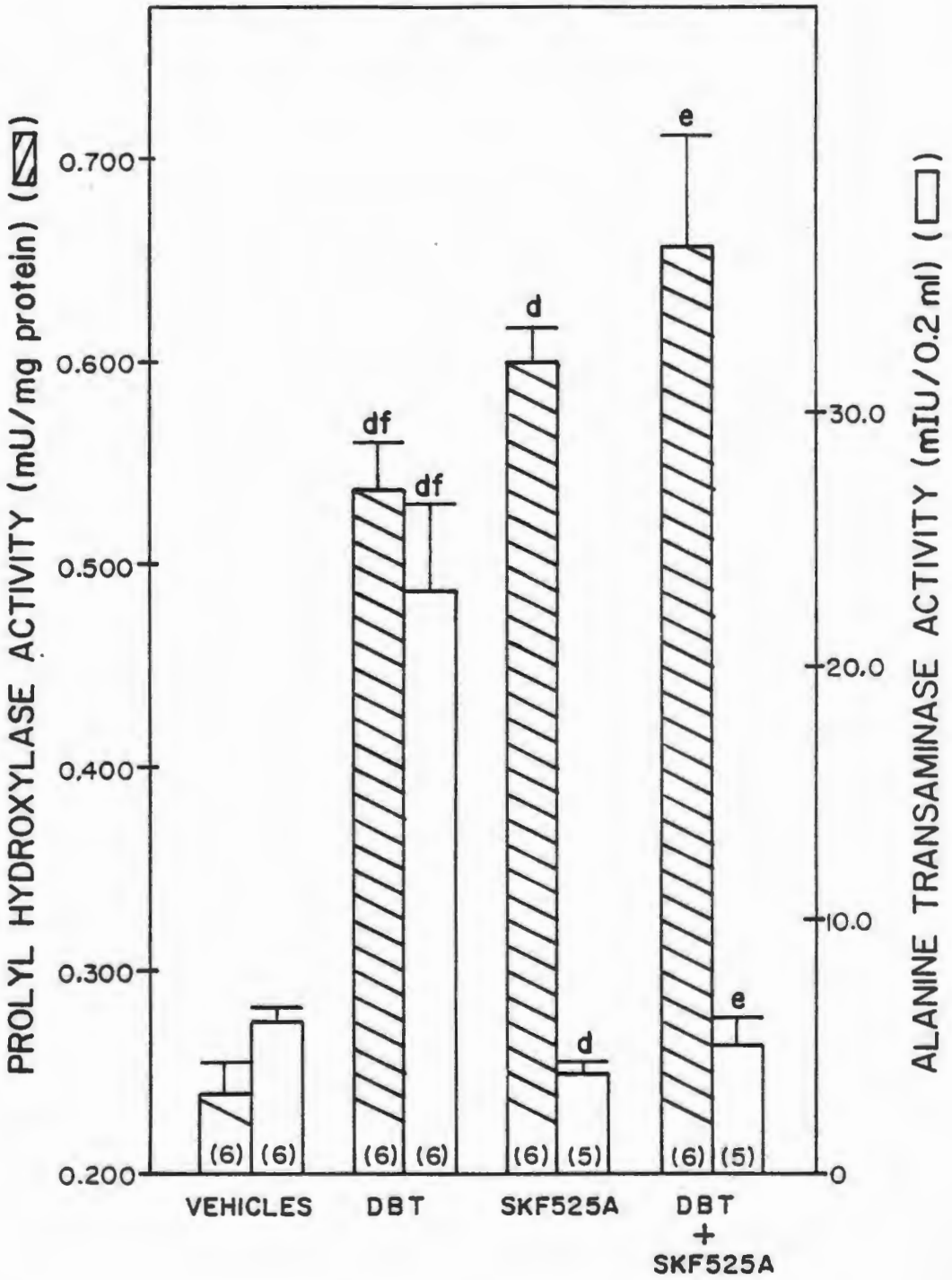
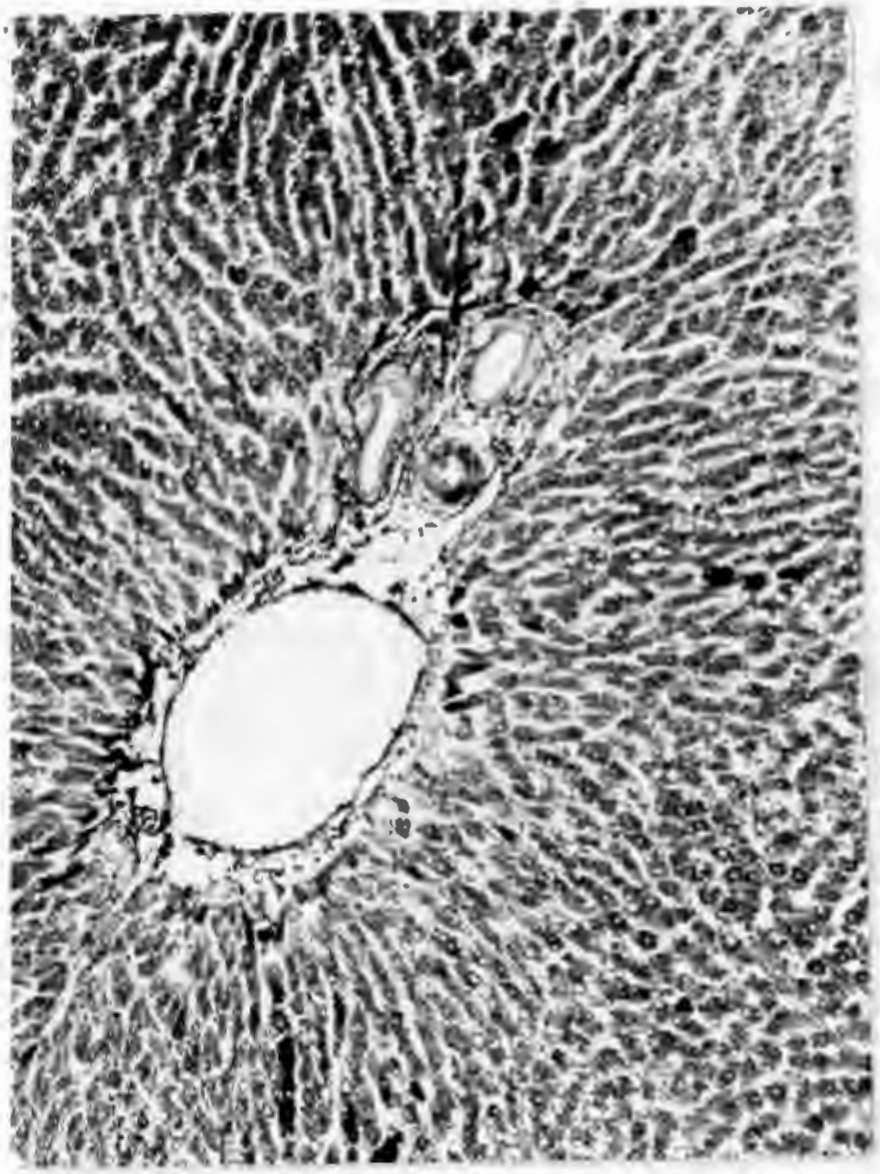

PROLYL HYDROXYLASE ACTIVITY (mU/mg protein) (▨)
ALANINE TRANSAMINASE ACTIVITY (mIU/0.2 ml) (□)
0.700
0.600
0.500
0.400
0.300
0.200
30.0
20.0
10.0
0
df
df
d
d
e
e
(6) (6)
(6) (6)
(6) (5)
(6) (5)
VEHICLES
DBT
SKF525A
DBT + SKF525A

Figure 14.

LIVER FROM A RAT TREATED WITH SKF 525A (50 mg/kg, b.i.d., i.p.) FOR 4 DAYS SHOWING FATTY INFILTRATION. TRICHROME STAIN x 100.



presence of fatty infiltration (Figure 15). Livers from rats treated with SKF 525A and DBT (Figure 16) were very similar to those in the SKF 525A group. Fatty infiltration was evident, but periportal inflammation and biliary injury seen with DBT treatment alone for 4 days (Figure 6b) were absent.

Figure 15.

FATTY INFILTRATION IN LIVER PARENCHYMAL CELLS AFTER 4 DAYS OF SKF ADMINISTRATION (50 mg/kg, b.i.d., i.p.). TRICHROME STAIN x 400.

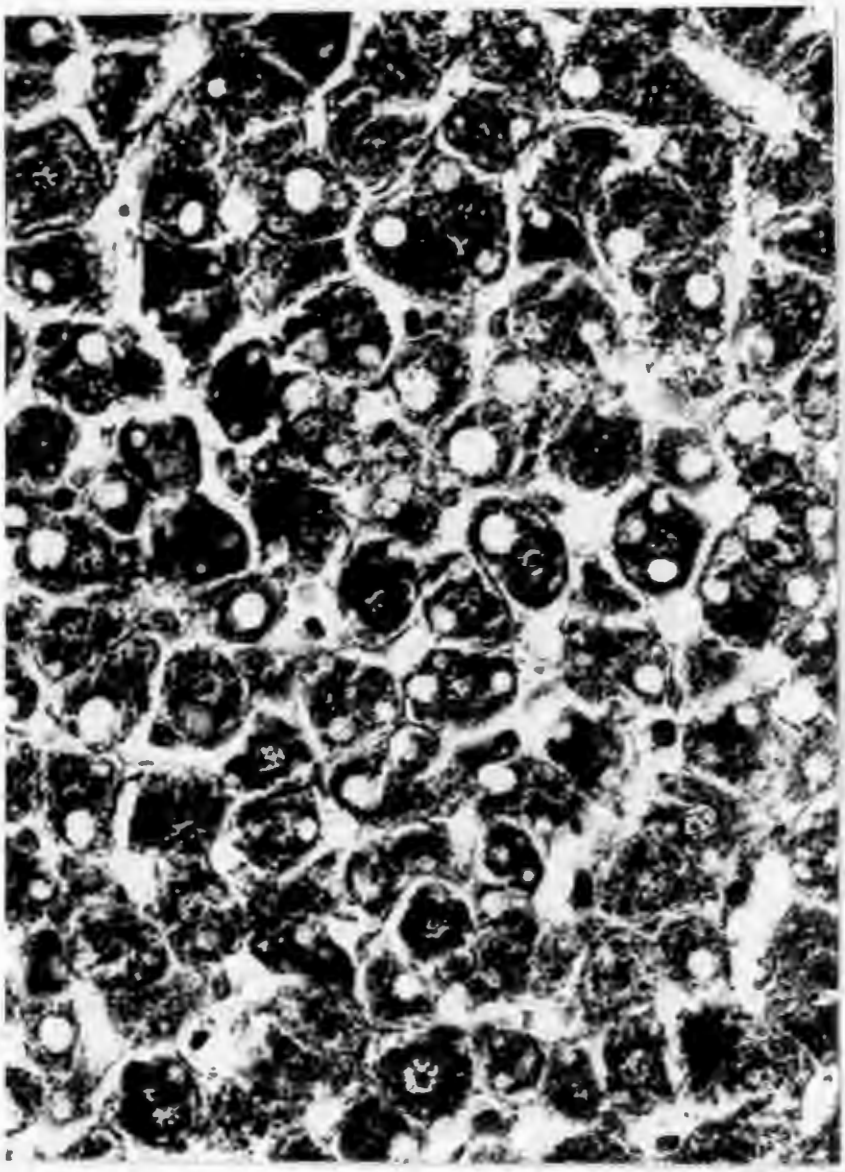
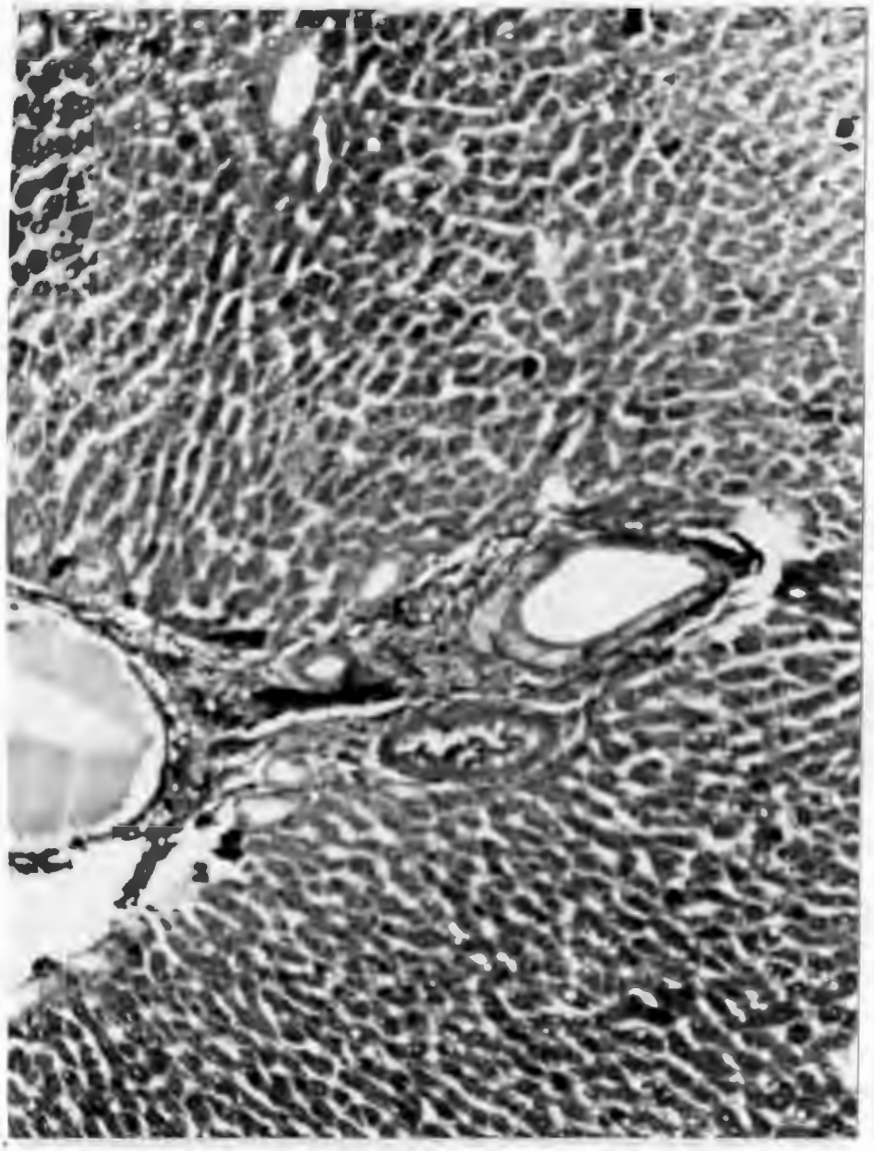


Figure 16.

PORTION OF A PORTAL TRACT IN RAT LIVER AFTER 12 DAYS OF DBT (20 mg/kg, q.o.d., i.p.) AND SKF 525A (50 mg/kg, b.i.d., i.p.) ADMINISTRATION. FATTY INFILTRATION CAN BE SEEN IN HEPATOCYTES. TRICHROME STAIN x 100.



DISCUSSION

Administration of DBT from 0 to 4 days produces a progressive increase in the two parameters of hepatic collagen synthesis examined, prolyl hydroxylase activity (PHA) (Figure 1) and *in vitro* collagen synthesis (Figure 2). The correlation (r=0.94) between these two parameters (Figure 3) substantiates the predictive value of PHA as an estimate of hepatic collagen synthesis. A similar correlation has been observed in human liver biopsies taken from patients exhibiting both normal and pathological histology (Mann *et al.*, 1979).

The increase in PHA and *in vitro* collagen biosynthesis from 0 to 4 days are associated with increasing frequency and severity of histopathologic changes during this period (Figures 4-6). The periportal inflammation, present throughout most specimens by day 4, contains predominantly polymorphonuclear cells and is, therefore, suggestive of acute inflammation. Proliferated bile ducts are often seen in the inflammatory area. Such proliferation is associated with many types of liver injury and is considered a characteristic feature of the fibrotic/cirrhotic process (Masuko *et al.*, 1964). Although histologic evidence of fibrosis in these livers is moderate at best, biochemical data indicate that fibrogenic processes are active after 4 days of DBT treatment.

Serum alanine transaminase is elevated approximately four-fold in rats treated with DBT for 4 days. Since increased serum alanine transaminase reflects parenchymal liver injury (Kachmar and Moss, 1976)

and such injury is very rarely observed in livers from DBT-treated rats unless it is proximal to inflammatory areas, this increase in serum enzyme activity is probably a function of the inflammatory process in the liver.

After 12 days of DBT administration, increases in hepatic PHA, in the percent of *in vitro* protein synthesis directed toward collagen, and in liver hydroxyproline content are observed (Table 2). The increase in the activity of prolyl hydroxylase is less than that observed after 4 days of DBT treatment (146% vs. 221%). This is not indicative of reversal of liver injury. Rather, PHA has been shown to be an early indicator of increased hepatic collagen synthesis both in human liver disease (Mann *et al.*, 1979) and in carbon tetrachloride-induced experimental liver injury (Lindblad and Fuller, 1980).

The approximately two-fold increase in the percent of labelled protein digested by collagenase appears to be the result of a small increase in the amount of label released from liver protein by collagenase relative to decreased incorporation into collagenase-resistant protein; the changes in these latter two parameters, when considered alone, are not statistically significant. The increase in histologically demonstrable fibrosis after 12 days of DBT administration suggests that the increase in the percent *in vitro* collagen synthesis is due to replacement of viable parenchyma with areas of connective tissue and collagen-producing cells. Such connective tissue accumulation is also apparent from the increased hydroxyproline content in these livers.

Neither of the serum enzyme activities measured in rats after 12 days of DBT administration are significantly elevated above control values (Table 3). Since serum alanine transaminase activity usually reaches peak levels early in liver injury (Kachmar and Moss, 1976), the low levels of this enzyme at this time are not surprising.

Large increases in serum alkaline phosphatase are observed in both humans (DeRitis et al., 1965) and rats (Kaplan and Righetti, 1970) as a result of cholestasis. This increase in serum alkaline phosphatase is thought to be a result of de novo synthesis by the liver of an isoenzyme of hepatic alkaline phosphatase (Kaplan and Righetti, 1970). Since alkaline phosphatase is not elevated in serum from rats administered DBT for 12 days (Table 3), cholestasis does not appear to be a factor in the cause and/or perpetuation of DBT-induced liver injury at this time. Since this enzyme is inhibited by certain heavy metals (Kachmar and Moss, 1976), in vitro inhibition of alkaline phosphatase activity in control serum by DBT was investigated to ascertain whether such an effect could be responsible for erroneously low enzyme activity in DBT-treated rats; no such inhibition could be demonstrated.

Liver histopathology after 12 days of DBT administration (Figures 7 and 8) reveals extensive periportal inflammation; both mononuclear and polymorphonuclear cells are present. Ductular cell injury and proliferation and fibrosis within the portal tracts are extensive. The central veins and parenchyma proximal to them remain well preserved. Although the majority of DBT-treated rats exhibit such a periportal lesion, a small number of animals invariably fail to do so. On occasion, granulomatous lesions are observed in livers

after 12 days of DBT treatment.

The role of inflammation in DBT-induced fibrogenesis was investigated with naproxen, a compound which has been shown to exhibit potent antiinflammatory and antigranuloma activity (Rook II, 1970). The effect of naproxen, administered alone and in combination with DBT for 12 days, is shown in Figure 9. As determined by a multifactorial analysis of variance with the treatment variables DBT and naproxen, there is no significant interactive effect with respect to PHA. However, when the naproxen group is compared to the naproxen/DBT group in a one-way analysis of variance, there is no significant difference between them. This suggests that DBT's fibrogenic properties are modified in the presence of naproxen, but that the effects of this antiinflammatory drug are moderate at best. There is no significant increase in serum alanine transaminase in any group. The decrease observed in the naproxen group is not meaningful on a physiological level.

DBT-induced toxicity is similar in many respects to the clinical entity, primary biliary cirrhosis (PBC). PBC is a chronic liver disease typified histologically by destructive cholangitis. As such, the term PBC is a misnomer but has been adhered to by convention. PBC has been categorized as an autoimmune disease by virtue of antiductular cell (Paronetto et al., 1964a and 1964b) and antimitochondrial (Dickson et al., 1979) antibodies present in the liver. However, an autoimmune etiology is by no means established.

The histological appearance of DBT-induced fibrosis, like PBC (Rubin et al., 1965), begins with periportal inflammation and bile

duct proliferation and progresses to destructive cholangitis and, finally, to fibrosis in its later stages. PHA is increased in PBC prior to histologically demonstrable collagen accumulation (Jain et al., 1978) as is seen in DBT-injured livers. Impaired cell-mediated immunity has been associated with PBC (MacSween and Thomas, 1973; Schaffner, 1979) and is produced by the administration of DBT to rats (Seinen et al., 1977a and 1977b). Finally, it has been suggested that PBC is drug-induced since this disease is associated with a history of drug hypersensitivity (Paronetto, 1964a).

The similarities between PBC and DBT-induced liver injury and the immunologic alterations in the former raised the suspicion that DBT's effects on the liver might have an immunologic component. The invariable failure of a small number of DBT-treated rats to respond with the hepatic lesions typical of DBT exposure, also suggested a host-dependent factor in DBT-induced injury.

Since DBT administration to neonatal rats impairs cell-mediated immunity and produces thymus atrophy (Seinen et al., 1977a and 1977b) and thymotoxic compounds are less effective in adult animals, hepatic injury produced by DBT was compared in various age groups. This was to serve as a starting point in the investigation of a possible relationahip between impaired thymus-dependent immunity and liver injury. DBT administration for 12 days to rats 14 weeks of age at the start of treatment produces periportal fibrosis similar to that described above for rats 9 weeks of age. However, the 4-week-old group is relatively resistant. In this latter group, PHA is elevated by 125% of control as compared to 146% and 215% in the 9 and 14 week-old

groups, respectively (Table 4). Leucocytosis (Table 5) and weight loss (Figure 12), usually associated with DBT-induced liver injury, are absent in these younger rats. Liver histology in these 4-week-old rats is similar to that seen in control animals (Figure 11).

The age-dependent effects of DBT strengthened the suspicion that DBT-induced liver injury may have an immunologic component and, specifically, one that is thymus-dependent. When thymus weights were measured in 4-week-old rats treated with DBT for 12 days (Table 6), it became apparent that DBT produces thymotoxicity in these animals similar to that reported in the literature for neonatal rats (Seinen et al., 1977a and 1977b). This thymus atrophy can not be attributed to dehydration since the effect is equally pronounced expressed as wet or dry weight.

To determine whether the inverse relationship between DBT-induced thymotoxicity and liver injury is related to a thymus-dependent immunologic component in the latter, a number of immunologic studies were performed on the 9-week-old rats. The assay for lectin-dependent lymphocyte-mediated cytolysis shows no difference between control and DBT-treated rats indicating that cytotoxic lymphocytes are not associated with DBT-induced liver injury.

The test for delayed-type hypersensitivity (Table 7) shows a characteristic foot swelling subsequent to a challenge dose of DBT in both control rats and those administered DBT for 12 days. This suggests that an immunologic reaction is not responsible. Histological observation of feet from control and DBT-treated rats, both of which were challenged with DBT, reveals no apparent differences.

However, striking differences are observed in rats challenged with DBT (Figure 12b) or DBT/liver compared to vehicle (Figure 12a) or liver. The predominantly polymorphonuclear cell infiltration in Figure 12b is indicative of acute inflammation.

Since thymotoxicity could impair humoral immunity through t-helper cells, direct immunofluorescence was used to look for the presence of antibodies directed against DBT and/or hepatic antigens in livers of DBT-treated rats. The immunofluorescence observed was localized to, but of uniform intensity throughout, portal tracts. It is suggestive of a reaction against serum IgG, which is likely to be increased periportally due to the inflammation. The immunofluorescence is not intensified at a given cell type or area within the portal tract.

No evidence for immunologic processes in DBT-induced liver injury is provided by these experiments. However, one can only conclude from these studies that an immunologic component of DBT-induced liver injury is not apparent at this time. If the duration of DBT exposure in rats could be expanded to more closely approximate drug-induced liver disease in humans, immunologic processes might become evident. One must consider that the immunologic processes seen in association with destructive cholangitis in human liver disease are usually investigated in late stages of the disease. This is due to both the lack of availability of liver biopsies in early stages of human liver disease for ethical reasons and the insidious onset of PBC (Dickson et al., 1979). Also, the immunologic phenomena are generally beleived to be reactive rather than eausative in destructive

cholangitis (Popper and Schaffner, 1970) and, therefore, would be expected to appear in more advanced stages of such liver disease.

Since the metabolic capabilities of the hepatic microsomal monooxygenase system increase with maturity (Niems et al., 1976) and DBT has been shown to be metabolized by this system in rats (Kimmel et al., 1977), the relationship of possible differences in such metabolism to the age-dependent effects of DBT was investigated. An inhibitor of hepatic microsomal enzymes, SKF 525A, was administered alone and concomitantly with DBT for 4 days. The effects of such treatments on PHA and serum alanine transaminase are shown in Figure 13. DBT-induced elevation of alanine transaminase is completely inhibited by SKF 525A.

PHA is elevated in both the DBT and SKF 525A groups. However, PHA in the DBT/SKF 525A group is less than what would be expected if the effect of these drugs on this enzyme are additive. Indeed, the interactive effect is significant as determined by a multifactorial analysis of variance with the treatment variables DBT and SKF 525A. This interaction is not likely to be a result of threshold prolyl hydroxylase activation since higher levels (4-fold) have been observed in experimental liver injury after 4 days of treatment with dimethylnitrosamine (Ristelli et al., 1978). The prolyl hydroxylase values in DBT/SKF 525A-treated rats are not erroneously low due to altered liver protein content since there is no change in the latter in any group. There is a positive correlation between PHA and serum alanine transaminase values in the control and DBT groups ($r=+0.83$). However, the correlation between these two parameters in the control

and SKF 525A groups is negative (r=-0.81). This suggests that DBT-induced PHA elevation is associated with parenchymal liver injury, and that of SKF 525A is independent of such injury and caused at least in part by a different mechanism.

Periportal inflammation and biliary injury, usually seen in DBT-treated rats after 4 days (Figure 6b), are absent in livers from rats that received SKF 525A in combination with DBT (Figure 16). Livers from this group of animals differ from control only in the presence of fatty infiltration. Livers from SKF 525A-treated rats are similar to those in the DBT/SKF 525A group; portal tracts show no evidence of fibrosis despite elevated PHA (Figure 14), but fatty infiltration is apparent throughout the parenchyma (Figure 15).

SKF 525A has been shown to inhibit microsomal drug metabolizing enzymes (Niems et al., 1976), to prevent biliary excretion of xenobiotics through its effect on the metabolism of these compounds (Levine et al., 1970), and to prevent gastrointestinal absorption of certain drugs (McLean and Marchang, 1970). Therefore, each of these effects must be considered as possible contributions to the pharmacologic interaction between DBT and SKF 525A.

Microsomal oxidation of alkyltin compounds produces hydroxylated metabolites, some of which are highly unstable and rapidly destannylated (Kimmel et al., 1977) and some of which retain their biological activity (Aldridge et al., 1977). In the case of tributyltin metabolites, which have been extensively studied, one has been shown to possess increased potency with respect to one of the parent compound's toxic effects, but the remaining four identified meta-

bolites are either highly unstable or decreased in biologic activity (Aldridge et al., 1977). The product of DBT destannylation, monobutyltin, has been reported to be relatively devoid of toxic effects (Barnes and Magos, 1968). Thus, for microsomal metabolism to produce an overall increase in liver toxicity, the more stable metabolites of DBT would have to exhibit rather potent toxicity to compensate for the relative inertness of monoalkyltin. Since there is no evidence that metabolism of tri- or dialkyltin compounds substantially increases their toxicity, it is unlikely that the protective effect of SKF 525A is due to decreased production of toxic metabolites.

Metabolism of DBT would be likely to increase the biliary excretion of this compound for two reasons. Firstly, metabolites would be likely to possess a more polar group thereby enhancing excretion (Kimmel et al., 1977; Levine, 1978). Secondly, microsomal metabolism would probably result in metabolites of increased molecular weight bringing the molecular weight of DBT (M.W. 304) above the approximate lower threshold value of between 200-300 (Levine, 1978). Thus, it seems likely that inhibition of metabolism by SKF 525A could decrease the biliary excretion of DBT and/or its metabolites.

Finally, SKF 525A has been reported to impair gastrointestinal absorption of various drugs (McLean and Marchand, 1970). This effect is tempory, however. After one hour (one hour after drug administration and 1.5 hours after SKF 525A administration), blood levels of the drugs examined were actually significantly greater in SKF 525A-treated rats compared to controls. With respect to DBT, 10 mg/kg/day, p.o. administered to rats has been shown to increase PHA significantly

by 4 days time (Yermakoff, 1978). Therefore, even if up to 50% of the administered dose was not absorbed, an elevation of liver PHA would still be anticipated in the presence of SKF 525A. It does not seem likely, therefore, that decreased gastrointestinal absorption of DBT by SKF 525A contributes substantially to the protective effects of the latter.

In summary, the pharmacologic interaction between DBT and SKF 525A may be due to multiple effects produced by SKF 525A including decreased production of toxic metabolites, decreased biliary excretion of DBT metabolites, and impaired gastrointestinal absorption of DBT. However, available information suggests that impaired biliary excretion via SKF 525A's inhibition of microsomal metabolism of DBT is likely to contribute substantially to the protective effect. Also, protection against DBT-induced liver injury by this inhibitor via its effects on microsomal metabolism may explain the age-related effects produced by this organotin compound.

CONCLUSIONS

(1) DBT administration from 0 to 4 days produces a gradual increase in prolyl hydroxylase activity (PHA) and in vitro collagen synthesis. These two parameters are significantly correlated (r=0.94). The biochemical changes are associated with the increasing appearance of inflammatory cells and ductular cell proliferation and injury during this same period.

(2) At 4 days after DBT administration, serum alanine transaminase is elevated approximately 4-fold. This is probably a sequela to the periportal inflammation since the hepatic injury observed at any time during the course of DBT treatment is associated with inflammation.

(3) Twelve days of DBT treatment (20 mg/kg, q.o.d.) results in increased PHA, increased protein synthesis directed toward collagen, and an increase in hydroxyproline content. These biochemical changes are associated with histologically demonstrable periportal fibrosis.

(4) Serum alanine transaminase and alkaline phosphatase activities are not elevated after 12 days of DBT administration. The return to normal levels of serum alanine transaminase from the 4-fold increase at 4 days time is not unusual since this enzyme generally peaks early in the course of tissue injury. Normal alkaline phosphatase levels indicate that cholestasis is not a factor in DBT-induced liver

injury at this time.

(5) Histopathology after 12 days of DBT treatment reveals extensive periportal inflammation and biliary injury in association with fibrosis. The contribution of inflammation to the development of fibrosis is supported by the modification of DBT-induced PHA elevation by the nonsteroidal antiinflammatory drug, naproxen.

(6) Rats 4 weeks of age are relatively resistant to the effects of DBT. In this 4-week-old group, PHA was 125% of control compared to 146% and 215% in the 9 and 14 week groups, respectively. Leucocytosis and weight loss, usually associated with DBT-induced liver injury, are absent in these younger rats.

(7) A thymocyte-dependent immunologic component of DBT's effects on the liver in the 9 and 14 week-old groups is suggested by the relative resistance of these 4-week-old rats with thymus atrophy to liver injury. Investigation of lymphocyte-mediated cytolysis, delayed-type hypersensitivity, and immunofluorescence in DBT-treated rats failed to reveal such an immunologic component.

(8) The relationship of differences in hepatic microsomal metabolism to the age-related effects of DBT was investigated using SKF 525A. There was a significant interaction with respect to the effects of DBT and SKF 525A on PHA. Administration of SKF 525A with DBT completely inhibited the increase in serum alanine transaminase seen with DBT alone after 4 days of treatment. Periportal inflammation

and biliary changes seen with DBT treatment alone were absent in these rats. Although the effects of SKF 525A may be due, in part, to the absence of DBT metabolites with greater biologic activity or impaired absorption of this organotin, they are more likely to involve decreased biliary excretion of the DBT and/or its metabolites. The interaction of SKF 525A with DBT suggests decreased biliary excretion via metabolism as a factor in the age-dependent effects produced by this organotin compound.

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