The effect of probucol on ethanol induced matrix changes in relationship to cirrhosis of liver

Ravi Gadi
The University of Montana

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Date: 05/23/94

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THE EFFECT OF PROBUCOL ON ETHANOL INDUCED MATRIX CHANGES IN RELATIONSHIP TO CIRRHOSIS OF LIVER

By
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Presented in partial fulfillment of the requirement for the Degree of
Master of Science in Pharmaceutical Sciences
University of Montana

1994

Approved by:

[Signatures]
Chairman, Board of Examiners
Dean, Graduate School

May 25, 1994
Date
Cirrhosis is characterized by a build-up of collagen in the liver. Many laboratories have demonstrated the stimulatory effect of chronic ethanol intoxication on collagen levels in the liver. However, the mechanism of the stimulatory effect of ethanol is not known and appears to be related to the metabolite, acetaldehyde. Acetaldehyde is a relatively reactive aldehyde that can adduct onto proteins, such as collagen, and possibly generate free radicals. The generation of free radicals may cause a build-up of collagen and may produce extracellular matrix changes.

The purpose of this project was to determine whether a potent antioxidant, probucol, could prevent collagen changes and the initial stages of alcoholic cirrhosis. Sprague Dawley rats (28) were divided into four treatment groups; control, ethanol-treated, probucol-ethanol-treated, and probucol-treated. After five months of treatments the rats were sacrificed and the livers were excised for analyses. Collagen content, lipid peroxidation, lipid levels, advanced glycated product levels, and collagenase digestibility were measured in each liver.

Those rats which were treated with ethanol had increases in collagen content, while advanced glycated products and collagen digestibility were decreased in the ethanol-treated animals. Concurrent administration of probucol, a potent antioxidant, significantly decreased the collagen content to a level similar to those in controls. It also decreased the advanced glycated product levels and the collagenase-insoluble fraction, suggesting that free radical generation may, somehow, be involved in the process of crosslinking of proteins, particularly collagen.

The pathological evaluation showed the presence of necrosis in ethanol-treated groups. However, no necrosis was observable in the probucol-treated groups.

These results indicate that oxidative stress may play an important role in the development of ethanol-induced cirrhosis of the liver. The data also suggest that some advanced glycated products are generated by an oxidative stress mechanism.
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<th>Description</th>
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<tr>
<td>ADH</td>
<td>Alcohol dehydrogenase</td>
</tr>
<tr>
<td>AGP</td>
<td>Advanced glycated products</td>
</tr>
<tr>
<td>ALDH</td>
<td>Acetaldehyde dehydrogenase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>Clg</td>
<td>Cold insoluble globulin</td>
</tr>
<tr>
<td>CONFI. INT.</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGP</td>
<td>Early glycated products</td>
</tr>
<tr>
<td>FDL</td>
<td>Freeze dried liver</td>
</tr>
<tr>
<td>FSC</td>
<td>Fat storing cells</td>
</tr>
<tr>
<td>FU</td>
<td>Fluorescence units</td>
</tr>
<tr>
<td>HP</td>
<td>Hydroxyproline</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LP</td>
<td>Lipid peroxidation</td>
</tr>
<tr>
<td>MEOS</td>
<td>Mitochondrial ethanol oxidizing system</td>
</tr>
<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide (reduced)</td>
</tr>
<tr>
<td>PROS</td>
<td>Partially reduced oxygen species</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
</tbody>
</table>
TBA .......................... Thiobarbituric acid
TBARS ......................... Thiobarbituric acid reactive substances
TCA ............................. Tricarboxylic acid
TCAA ............................ Trichloroacetic acid
VLDL ............................ Very low density lipoprotein
ACKNOWLEDGEMENTS

I would like to thank the Dean, faculty and staff of the School of Pharmacy for their educational and financial support that enabled me to attain this milestone in my life. I am also grateful to my major advisor Dr. Bryan D. Tarr for his patience, friendly guidance, and continued encouragement throughout the course of this research.

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INTRODUCTION

CIRRHOSIS OF LIVER

Ethyl alcohol (ethanol) is the oldest mood modifying drug known to man with its social and clinical abuse recorded in antiquity. Ethyl alcohol dependence was defined by the World Health Organization (WHO) in 1952 as occurring in those "excessive drinkers whose dependence on ethanol has attained such a degree that it shows a noticeable mental disturbance or interference with mental or bodily health, interpersonal relations and their smooth social and economic functioning or who show the prodromal signs of such development". The acute ingestion of ethanol even in intoxicating doses, probably produces transient change in hepatic function. However, Leiber noted that ethanol, if consumed on a regular basis, produces a constellation of dose related deleterious effects that appear to result principally from its metabolism (Lieber, 1988a, 1988b).

When chronic diseases cause the liver to become permanently injured and scarred, the condition is called cirrhosis. The scarred tissue formed in cirrhosis harms the structure of the liver, blocking the flow of blood through the organ. The loss of normal liver tissue slows the processing of nutrients,
hormones, drugs, and toxins by the liver.

About 25,000 people die from cirrhosis each year in the United States alone. There also is a great toll in terms of human suffering, hospital costs, and the loss of work by people with cirrhosis. In the United States, chronic alcoholism is the most common cause of cirrhosis. Cirrhosis can also be caused by diseases such as chronic viral hepatitis (types B, C, and D) or may result from injury due to a number of inherited diseases such as cystic fibrosis, alpha-1 antitrypsin deficiency, hemochromatosis, Wilson's disease, galactosemia, and glycogen storage diseases (NIH Publication 92-1134, 1991).

The accumulation of fat in the liver is an early event and can occur in normal individuals after the ingestion of relatively small amounts of ethanol. This results from inhibition of the tricarboxylic acid cycle (TCA) and the oxidation of fat is, in part due to the generation of an excess amount of reduced nicotinamide adenine dinucleotide (NADH) produced in the hepatic cytosol as a result of alcohol dehydrogenase (ADH) mediated oxidation of ethanol. The regular ingestion of more than moderate amounts of ethanol leads to increased accumulation of acetaldehyde, probably due to reduced activity of acetaldehyde dehydrogenase (ALDH) (Jenkins and Peters, 1980; Palmer and Jenkins, 1985). Acetaldehyde is thought to cause a host of deleterious effects, including damage to mitochondrial and other cellular membranes, enhanced lipid peroxidation and depletion of vitamins and trace metals. Such an insult causes the engorging of hepatocytes with protein, fat and water that progresses to the
necrosis and fibrosis found in cirrhotic liver observed in alcoholic individuals.

Alcoholic Cirrhosis of Liver

In 1986, cirrhosis was ranked as the ninth leading cause of death in this country (NIAAA, 1989). There are differing levels of susceptibility to the medical consequences of ethanol consumption. Some people may exhibit a greater predisposition to develop adverse medical consequences from ethanol use, either for genetic or other reasons. The effect of ethanol consumption on various organs may either be direct or indirect. Moreover, there appears to be a relationship between the development of medical consequences and the amount, duration, and pattern of ethanol consumption which may either be sporadic or continuous.

Ethanol-induced liver damage is grouped under three major headings: (1) fatty liver, (2) alcoholic hepatitis, and (3) cirrhosis. Histologic evidence of all three can be found simultaneously in the same patient (Maddrey, 1988). The three types of ethanol-induced liver disorders differ in their prognoses: Fatty liver (steatosis) and alcoholic hepatitis are reversible with abstinence, whereas cirrhosis is not. They also differ in estimates of their incidence. The majority of the heavy drinkers (90 to 100 percent) show evidence of some features of fatty liver, of which an estimated 10 to 35 percent develop alcoholic hepatitis, and 10 to 20 percent develop cirrhosis (Sorenson et al, 1984, Grant et al, 1988). However, Leiber noted that it has
been estimated from autopsy studies that 40 percent of cirrhosis is not detected during life (Lieber, 1982).

Factors leading to development and progression of cirrhosis

A. GENDER

Cirrhosis death rates for males have been consistently higher than death rates for females. In 1986, the male cirrhosis mortality rate was more than twice the rate for females (NIAAA 1989). However, women appear to have greater susceptibility to ethanol-related liver damage (Table 1). They develop severe liver disease with shorter durations of ethyl alcohol use and lower levels of consumption per kg bodyweight (Grant and coworkers, 1988). Further, surveys have shown a higher prevalence and greater severity of ethanol-related liver disease in ethanol-dependent females than in ethanol-dependent males (Marbet, 1987). The reasons for the apparent sex differences in ethanol sensitivity are unknown. They could be related to differences in hormones, immune systems, body weight, or body water content (Grant and coworkers, 1988).
<table>
<thead>
<tr>
<th></th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cumulative ethyl alcohol consumption</td>
<td>917 kg</td>
<td>570 kg</td>
</tr>
<tr>
<td>Percent of dose consumed by males</td>
<td>100%</td>
<td>62%</td>
</tr>
<tr>
<td>Percentage of developing cirrhosis</td>
<td>33%</td>
<td>63%</td>
</tr>
</tbody>
</table>

\(^{a}\) modified from Marbet et al, 1987  
\(^{b}\) All patients were drinking a minimum of 40 g ethyl alcohol per day for 6-8 years.
B. RACE

The cirrhosis mortality rate in the United States was consistently higher for whites than for nonwhites between years 1937 through 1956 (NIAAA, 1989). Beginning in 1957, mortality rate as a result of cirrhosis in nonwhites rose sharply, peaking at 25.3 deaths per 100,000 in 1972; nearly twice the rate for whites. For statistical purposes, hispanics were categorized as white, and blacks comprise approximately 92 percent of the non-white population. Therefore, data on nonwhites is considered reasonable representative of the black population.

In the United States, striking changes have occurred over time in mortality rates as a result of cirrhosis for nonwhite males. For nonwhite males, cirrhosis death rates were consistently lower than for white males from 1933 until 1960. After 1960, the rates for nonwhite males rose steeply and by 1969, the mortality rate for nonwhite males had nearly doubled the 1960 rate. In 1972, nonwhite male mortality peaked at 35 deaths per 100,000 and has declined rapidly since then (NIAAA, 1989). The mortality rate for nonwhite females followed a similar pattern, rising steadily above the white female rate from the mid-1950s until 1973, when it peaked at more than twice the rate for white females. However, in the 65-and-over age group, white males and females consistently have higher respective cirrhosis mortality rates than nonwhite males and females (NIAAA, 1989).
C. AMOUNT AND DURATION OF ETHANOL CONSUMPTION

The amount of ethanol consumed and the duration of ethanol use that leads to liver injury is uncertain. It has been shown, that it is possible to induce fat accumulation and ultrastructural changes in the liver of young, nonalcoholic volunteers in about a week by substituting ethyl alcohol for carbohydrate in the diet in amounts well below those needed to induce intoxication (Rubin and Lieber, 1968). However, there are marked individual differences in susceptibility to ethanol-induced liver disease (Maddrey, 1988) as well as international differences in the relationship between per capita ethyl alcohol consumption and cirrhosis mortality rates. Genetic factors may be responsible for the differences, not at the crude HLA histocompatibility antigen level, but rather via genetic polymorphism of the two enzyme systems: microsomal-enzyme-oxidizing system (MEOS) and alcohol dehydrogenase (ADH) which metabolize ethyl alcohol (Bosron, 1986). Grant and coworkers found that there exists an increased risk of fatty acid liver at consumption levels greater than 80 grams a day in men and 20 grams a day in women (Grant et al, 1988). Several studies, but not all (Sorenson et al, 1984), have found that the severity of liver damage increases with increasing daily intake of ethyl alcohol to more than 180 grams per day (approximately 14 standard drinks) when duration of use ranges from 10 to 20 years (Grant et al, 1988). Leibach (1974) showed that for an individual consuming about 210 grams of ethanol daily for 22 years, the
probability of developing cirrhosis is 50 percent, and that it increases to 80 percent after 33 years of similar daily consumption.
LIVER AND EXTRACELLULAR MATRIX

The liver composition, especially the extracellular matrix (ECM), changes as the liver progresses from steatosis to complete cirrhotic liver. The connective tissue could provide specific signals for modulation of the phenotypic expression of cells, and accordingly it may play an important role in the development of alcoholic cirrhosis of liver.

Liver Composition

Morphometric analyses of the liver has been performed by two different groups with almost identical results. Between 78-84% of the total liver volume is occupied by the different cellular components and only 16-22% by the elements of the extra-cellular space (Blouin et al, 1977; Van de Werve, 1980). The space of Disse and the sinusoidal lumen account for most of the extracellular space (97.4%) and are freely interconnected with each other through the fenestration of the endothelial cells (see Table 2). Although there is no morphologically detectable basement membrane between the sinusoidal lining and the hepatocytic plates, some macromolecules, normally found in basement membranes, have been detected by an immunocytochemical methods (Hahn et al, 1980; Biempica et al, 1980; Voss et al, 1980 and Gay, 1980).
<table>
<thead>
<tr>
<th>Component</th>
<th>Composition (Percent)</th>
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<tr>
<td><strong>I. Cells</strong></td>
<td>84(^b) - 78(^c)</td>
</tr>
<tr>
<td>1. Hepatocytes</td>
<td>78.0 - 72.0</td>
</tr>
<tr>
<td>2. Non-Parenchymal</td>
<td>6.3 - 5.8</td>
</tr>
<tr>
<td>a. Endothelial</td>
<td>2.8 - 2.6</td>
</tr>
<tr>
<td>b. Kupffer</td>
<td>2.1 - 2.0</td>
</tr>
<tr>
<td>c. Fat storing cells (FSC’s)</td>
<td>1.4 - 1.3</td>
</tr>
<tr>
<td><strong>II. Extracellular Space</strong></td>
<td>16.0 - 22.0</td>
</tr>
<tr>
<td>1. Disse</td>
<td>4.9</td>
</tr>
<tr>
<td>2. Sinusoidal Lumen</td>
<td>10.6</td>
</tr>
<tr>
<td>3. Biliary Canaliculi</td>
<td>0.4</td>
</tr>
<tr>
<td>4. Collagens</td>
<td>0.1(^d)</td>
</tr>
</tbody>
</table>

\(^{a}\) Modified from Rojkind and Ponce-Noyola (1982).
\(^{b}\) Data from Blouin et al (1977).
\(^{c}\) Data from Van de Werve (1980).
\(^{d}\) Data from Knook and Leeuw (1981).
Hepatocytes account for 92% of the cellular volume of the liver and the remaining volume is occupied by the non-parenchymal cells which includes Kupffer, Ito or fat storing cells (FSC's), endothelial and Pit cells. Except for Pit cells, whose function is unknown, all of the other cells have been shown to possess full capacity to produce one or more components of the ECM. The parenchymal cells can also synthesize one or more elements of the extracellular connective tissue (Rojkind and Dunn, 1979; Voss et al, 1979; Hata et al, 1980; Foider et al, 1980; Guzelian et al, 1981; Wahl et al, 1981).

Extracellular Space

The extracellular space of the liver is composed of a large variety of macromolecules. The macromolecules are elements of the ECM and participate in the structure, organization and function of tissue. These include collagens, non-collagenous glycoproteins, glycosaminoglycans and proteoglycans.

Extracellular matrix

The ECM elements can be divided into three main categories: collagens, which are the most abundant component; proteoglycans, which regulate tissue permeability, hydration and fiber size; and glycoproteins, which connect ECM components in various tissues (Geoffrey LG, 1987; Martin GR and Kleinman HK, 1985).
Collagen

A complete analysis of liver collagen has been performed only in human and rat liver (Seyer and coworkers, 1977; Rojkind and coworkers, 1979; Seyer, 1980). Although there are important qualitative and quantitative differences, the four normal genetic types of collagen identified in human liver, mainly types I, III, IV and V, have also been detected in rat liver. Researchers have found the concentration of collagen in human liver to be $5.5 \pm 1.6$ mg per g of fresh liver and in the rats, the concentration of collagen is only $0.91 \pm 0.15$ mg per g of fresh liver (Rojkind et al, 1979; Ehrinpreis et al, 1980) (see Table 3).

Type I Collagen

The concentration of type I collagen in the normal human liver is approximately $2.0$ mg per g of fresh tissue while in the rat liver has about 360-400 mcg/g of fresh liver (Seyer and coworkers, 1977; Rojkind and coworkers, 1979; Seyer, 1980). This collagen type corresponds to the thick collagen bundles of the liver and forms the dense connective tissue. Type I collagen is present in the liver capsule, in the stroma of the portal tracts and large vessels and around terminal venula areas. Occasionally, type I collagen fibers are also seen inside the liver lobule (Gay et al, 1975; Gay, 1980; Voss et al, 1980; Biempica et al, 1980; Grimlaud et al, 1980) (Table 4).
**Type III Collagen**

The concentration of type III collagen in human livers is about 2.0 mg per g of fresh tissue and the ratio of type I/type III collagen is one. In the rat, the concentration of type III collagen is only 360-400 mcg per g of fresh tissue and the ratio of type I/III is also one (Seyer et al, 1977; Rojkind et al, 1979; Seyer, 1980). Type III collagen corresponds to some, but not all of the reticulin fibers of the liver. It is mixed with type I collagen in the stroma of the large vessels and portal triads. Inside the liver parenchyma, it forms the very fine framework of the tissue (Gay et al, 1975; Gay, 1980; Voss et al, 1980; Biempoca et al, 1980; Grimaud et al, 1980) and has been found inside the space of Disse in close association with the processes of Ito cells (Kent et al, 1976 and Oikawa, 1979).
### TABLE 3. Collagen content in Normal and Cirrhotic Livers of Man and Rat*b.

<table>
<thead>
<tr>
<th></th>
<th>Human b mg/g</th>
<th>Rat c mg/g</th>
<th>Normal</th>
<th>Cirrhotic</th>
<th>Normal</th>
<th>Cirrhotic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total-Collagen</td>
<td>5.5 ± 1.6</td>
<td>30</td>
<td>0.91 ± 0.15</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type I</td>
<td>2.0</td>
<td>15.0</td>
<td>0.40</td>
<td>3.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type I-Trimer</td>
<td>0.05</td>
<td>0.9</td>
<td>0.03</td>
<td>0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type III</td>
<td>2.0</td>
<td>8.0</td>
<td>0.40</td>
<td>3.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type IV</td>
<td>0.5</td>
<td>7.0</td>
<td>0.06</td>
<td>0.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type V</td>
<td>0.9</td>
<td>-</td>
<td>0.05</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ratio III/I + III</td>
<td>47.3d</td>
<td>37.5d</td>
<td>41.3g</td>
<td>37.5d</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>54.2f</td>
<td>39.6f</td>
<td>51.1g</td>
<td>30.4f</td>
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<td></td>
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<tr>
<td>Ratio I/III</td>
<td>1.0h</td>
<td>1.87h</td>
<td>1.0i</td>
<td>0.94i</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Modified from Rojkind and Ponce-Noyola, 1982.

b Values are average and were obtained from data by Seyer et al., 1979 and Rojkind et al., 1979 and were calculated assuming that a normal liver contains 5.5 mg of collagen/g and a cirrhotic liver contains 30 mg of collagen/g.

c Values are average and were obtained from data by Seyer, 1980, and Rojkind and Ponce-Noyola, 1982 and were calculated assuming that a normal liver contains 0.91 mg of collagen/g and a cirrhotic liver contains 8 mg of collagen/g.

d Taken from Seyer et al., 1977. Ratios were determined by the CNBr method.

e Taken from Seyer, 1980. Ratios were determined by the CNBr method.

f Taken from Rojkind et al., 1979. Ratios were determined after solubilization of collagens with pepsin.

g Taken from Rojkind and Ponce-Noyola, 1982 (Values were obtained after solubilization of collagens with pepsin).


TABLE 4. Specific distribution of Protein Components in the liver as determined by Immunofluorescence.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Portal Triad</th>
<th>Terminal Venule</th>
<th>Parenchyma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stroma</td>
<td>Around Elements</td>
<td>Space of Disse</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Containing a Basement membrane</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rim around vein</td>
<td>Sinusoidal Lining</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>I</th>
<th>Forms dense connective tissue (thick collagen bundles)</th>
<th>Rim around vein</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>III</td>
<td>Present in reticulin fibers mixed with Type I</td>
<td>Rim around vein</td>
<td>Present in contact with processes of Ito cells or FSC's</td>
</tr>
<tr>
<td>IV</td>
<td>-</td>
<td>Rim around vein</td>
<td>Discontinuous fine fibrillar pattern</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>Follows the distribution of type IV collagen</td>
<td>Rim around vein</td>
<td>-</td>
</tr>
<tr>
<td>Laminin</td>
<td>-</td>
<td>Rim around vein</td>
<td>-</td>
</tr>
<tr>
<td>V</td>
<td>Small amounts</td>
<td>Present only around vascular structures</td>
<td>Spotty distribution</td>
</tr>
</tbody>
</table>

* Modified from Rojkind and Ponce-Noyola, 1982.

^ A dash indicates that the molecule is not present in that location.
Type IV Collagen

The concentration of type IV collagen in human liver is approximately 500 mcg per g of fresh liver and in the rat liver it is 50-60 mcg per g of fresh liver. Type IV collagen is present in sites containing a basement membrane. It has been found around arteries, lymphatic vessels, bile ducts and ductules and nerve endings. It is also present between the endothelial lining of the sinusoid and the liver plates. In this region the staining is discontinuous (Gay, 1980; Hahn et al, 1980a). It is important to stress that even though type IV collagen, fibronectin and other components normally present in basement membranes have been found, there exists no morphologically detectable basement membrane in the sinusoidal region (Wick et al, 1978; Hahn et al, 1980; Voss et al, 1980; Carlsson et al, 1981). The presence of two of the proteins found in basement membranes suggests that the other components such as laminin and glycosaminoglycans may be required for the physical organization of basement membrane between the sinusoidal lining and the liver plates. This is of special interest, because a true basement is formed in disease states, such as in liver cirrhosis, as a result of changes in the pattern of deposition of the components of the extracellular matrix (Schaffner and Popper, 1963; Hruban et al, 1974).
**Type V Collagen**

The concentration of type V collagen in normal human liver is approximately 800 to 900 mcg per g fresh tissue (Rojkind et al, 1979) and in the rat it is 40-50 mcg per g of liver (Rojkind and Ponce-Noyola, 1982). Type V collagen is present in many areas of the liver but has special distribution around vessels. It is present also along the sinusoidal surface of the hepatocyte.

**Glycoproteins**

Even though several glycoproteins are present in the ECM of the liver, no quantitative information about their concentration is available. Most of the information has been obtained through qualitative antibody staining (Rojkind and Ponce-Noyola, 1982).

**Fibronectin**

Two forms of fibronectin have been described in animal tissue. The soluble form, which is present in plasma at a concentration of 30 mg/100 ml (Mosesson and Umflee, 1970; Mosher and Williams, 1978), has been termed Cold-insoluble globulin (CIg). The other form is insoluble and is present in most tissues either as a component of the ECM or that of the plasma membrane of some cells (Pearlstein et al, 1980; Ruoslahti, 1981; Ruoslahti et al, 1981). Fibronectin is the major protein component of the plasma membrane of the
fibroblast. The concentration of fibronectin in the cell membrane varies with the cell cycle, increasing in G1 phase and decreasing during mitosis. In transformed cells, the fibronectin plasma membrane concentrations were found to be very low (Hynes and Bye, 1974).

Fibronectin contains several domains which are manifested by its ability to bind to cells, collagen, glucosaminoglycans and fibrin (Pearlstein et al, 1980; Ruoslahti et al, 1981; Ruoslahti, 1981; Kleinman et al, 1981a). It has been suggested that a transglutaminase (Factor XIIa) may be responsible for the binding of fibronectin to the other tissue components (Mosher, 1975; Mosher et al, 1979; Mosher et al, 1980). It has also been shown to be involved in several other biological processes. It is an attachment factor for fibroblast and kupffer cells in culture (Pearlstein et al, 1980 and Ruosalhti et al, 1981) and also participates in maintaining cell shape (Hynes et al, 1979 Rennard et al 1981). It is a chemotactic factor for fibroblast and appears to be involved in phagocytosis (Gudewicz et al, 1980; Doran et al, 1981 and Postlethwaite et al, 1981).

Laminin

Laminin is a component of basement membranes and was first isolated by Timpl and coworkers from a murine tumor producing large amounts of type IV collagen. It is a high molecular weight polymer of 800,000-daltons and it is made of subunits of 400,000- and 200,000-daltons joined by disulfide bonds.
crosslinks (Kleinman et al, 1981a). Laminin closely follows the distribution of type IV collagen in sites containing typical basement membranes in the liver (Hahn et al, 1980a; Carlsson et al, 1981). Laminin is absent from the sinusoidal lining. Further, the function of laminin is not known. However, it has been suggested that it may be involved in morphogenesis, cell differentiation and the arrangement of the liver plate during cell regeneration after hepatectomy (Chung et al, 1979; Ekbloom et al, 1980; Sakashita and Ruoslahti, 1980; Carlsson et al, 1981). It also helps in the attachment of epithelial cells to type IV collagen (Terranova et al, 1980; Kleinman et al, 1981a). It may also play a role in cell-cell interactions and in the binding of glycosaminoglycans (Sakashita et al, 1980).

Cells Involved in the Biosynthesis of the Extracellular Matrix

It was shown several years ago that cultures of a large variety of cells that contain the enzyme proline hydroxylase possess the ability to produce collagen (Goldberg and Green, 1968; Langness and Undenfriend, 1974). Recently, it has been shown that primary cultures of hepatocytes that maintain the capacity to synthesize albumin are also able to produce collagen, glycoaminoglycans, laminin and fibronectin (Guzelian and Diegelmann, 1979; Voss et al, 1979; Diegelman et al, 1980; Foidart et al, 1980; Ninomiya et al, 1980; Guzelian et al, 1981). Fibronectin has also been shown to be produced by macrophages (Alitalo et al, 1980) and kupffer cells (Wahl et al, 1981).
It is important to stress that cell cultures may resemble more closely a process of wound healing than a normal physiological state. Cells that have been separated from their matrix by mechanical or enzymatic methods will have to synthesize part or all the components of the matrix in order to assure their survival in culture. The amount and types of proteins usually produced by the cells in vivo may be regulated in part by the microenvironment of the cell. Under pathological conditions, if the microenvironment is modified, all the cells in the liver could participate in the remodeling process and/or in wound healing. It is not known which kind of liver cells are responsible for producing different types of collagen in vivo. However, production of types of collagens has been attributed to cells found in close proximity to a particular type of collagen (Kent et al, 1976) or by similarities to epithelial or mesenchymal cells found in extrahepatic tissue matrix which are known to produce collagens (Hahn et al, 1980b).

The Extracellular Matrix in Cirrhosis

The macromolecules that form the ECM of the liver follow a specific pattern of distribution and organization (Table 4). In cirrhotic liver the proportion of ECM with respect of the number of cells increases and the normal organization of the matrix is lost. Thick collagen bundles surround nodules of hepatocyte (Seyer et al, 1977; Rojkind et al, 1979; Seyer, 1980; Voss et al, 1980; Gay, 1980; Biempica et al, 1980; Grimaud et al, 1980; Hahn et al,
1980a) and all the macromolecules are found together in scarring areas. As a consequence of the disorganization of the cell’s matrix, true basement membranes may be formed (Schaffner and Popper, 1963; Hruban et al, 1974). This could further contribute to the pathophysiology of the disease, because normally, there is free flow of macromolecules between the endothelial lining and the space of Disse through fenestrations. The appearance of a basement membrane, with a higher selectivity in the flow-through of macromolecules, could also increase the vascular resistance and thus contribute to the portal hypertension observed in cirrhotic patients.

According to the classical definition of liver cirrhosis, the nodules of hepatocyte remaining between the broad bands of scar tissue show variable degrees of regeneration (Leevy et al, 1976). Several parameters have been used to characterize the regenerative state of the liver including the modifications in the adenyl cyclase system and plasma levels of insulin and glucagon and their receptors (Blazquez et al, 1975; Leffert et al, 1979a; Mourelle and Rubalcava, 1979; Mourelle and Rubalcava, 1981). However, those changes are not known to occur in carbon tetrachloride-induced cirrhotic liver (Mourelle et al, 1981).

The liver shrinks as a result of cell necrosis and collapse, as well as from active contraction of scar tissue by myofibroblasts (Bhathal, 1972; Gabbiani et al, 1976; Irle et al, 1980; Hahn et al, 1980b). Consequently, the decrease in liver size is more dramatic than the increase in content of extracellular matrix.
While the former decreases three-fold, the latter only increases two-fold.

Interrelationships between Inflammation and Fibrogenesis

Many chemical and biological agents produce liver disease leading to cirrhosis of the liver. Despite their different mechanisms, the scar formed in the liver is always the same (Rojkind et al, 1979) and the collagens produced have the same susceptibility towards digestion with homologous collagenases as normal liver collagen (Perez-Tamayo and Montfort, 1980). Furthermore, the composition of the scar in the liver with regard to collagen, is not different from that produced in the skin, and accordingly, type I collagen is the predominant type. The ratio of type I to type III collagen is higher in cirrhotic livers than in normal livers (Seyer et al, 1977; Rojkind et al, 1979; Rojkind, 1980). The presence of common mechanisms in scar formation suggests that the same interrelationships observed between inflammatory cells and fibroblasts during inflammation and wound healing may be taking place in the liver during active fibrogenesis (Wahl and Wahl, 1981).

The concept of ECM accumulation has been progressively changing from the static morphological result of tissue damage to a dynamic active process, and currently emphasis is being put on the interactions between the extracellular framework, cell membrane receptors, and cellular structures and activities (Biagini and Ballardini, 1989). The sequence of events that leads to the development of cirrhosis of the liver as a result of chronic ethanol
intoxication are increased fat accumulation (fatty liver) which progresses to hepatitis (i.e., inflammation of the liver) and eventually leads to irreversible hepatic necrosis and fibrosis. Development and persistence of hepatic fibrosis depends upon the relative rate and magnitude of collagen synthesis and degradation. The amount of collagen significantly increases in human cirrhotic liver and may reach values of up to 30 mg per g tissue (Rojkind, 1982; Rojkind, 1983) as compared to 5.5 to 7.1 mg per g tissue (wet weight) in normal livers (Seyer et al, 1979; Rojkind, 1979). Liver biopsy specimens taken from patients with alcoholic hepatitis and ethanol-induced cirrhosis also showed an increase in collagen biosynthesis (Chen and Leevy, 1975; Perier et al, 1984).

**Collagen biosynthesis in alcoholic cirrhosis**

Lieber and Feinman (1972) found that long term ethanol administration (7 and 14 months) caused collagen accumulation in livers of rats and baboons. Bankowski and Pawlicka (1989; 1987) reported that chronic intoxication of rats with ethanol resulted in an increase in prolyl hydroxylase activity and also distinct, progressive increase of $\delta^3$H-proline incorporation into collagen synthesized by liver. Chronic administration of ethanol significantly affects the metabolism of liver proteins and slightly inhibits the incorporation of proline into non-collagenous liver proteins while stimulating biosynthesis of collagen (Bankowski and Pawlicka, 1989).
An increase in collagen biosynthesis is observable after the second month of ethanol intoxication, but the collagen content remains unchanged. According to Kato and coworkers (1985) this discrepancy can be explained by an increased collagen degradation in the early stages of alcoholic cirrhosis. Furthermore, Mezey and coworkers (1977) also reported an increase in urinary excretion of collagen degradation products. An imbalance between the deposition and the removal of the collagen results in excessive accumulation of the connective tissue in the liver. Collagens and other components of the connective tissue such as glycosaminoglycans, fibronectin, laminin, elastin, and cells also undergo metabolic turnover (at different rates), and therefore it is likely that fibrosis is a consequence of a non-equilibrium between the synthesis and degradation of such components. Excessive deposition of collagen in the liver may result from increased synthesis, decreased degradation or a combination of these two processes that may occur either simultaneously or successively. Many studies have found that there is an increased collagenase activity in early hepatic fibroplasia, and this may be related to architectural remodeling. Afterwards, the activity returns to normal or to lower than normal levels in late fibrosis and in cirrhosis (Popper and Berk, 1984; Popper and Lieber, 1980; and Thannasi and coworkers, 1980).
MECHANISMS OF ETHANOL INDUCED FIBROGENESIS

Ethanol induced liver damage proves to be a good model for the study of fibrogenesis since hepatic fibrosis of this etiology is not spontaneously progressive. The mechanism involved in deposition of collagen in the liver in alcoholic cirrhosis is not known (Popper and Martin, 1982; Mezey, 1982). It has been suggested that ethanol-induced fibrogenesis may occur in the absence of any hepatocellular inflammation in baboons and humans (Popper and Leiber, 1980; Nakano and coworkers, 1982). This would indicate that mechanisms other than those involved in inflammation might be responsible for fibrosis. Lack of a stimulatory effect of ethanol on collagen biosynthesis in cultured tissues such as human liver, fetal and skin fibroblasts (Galambos and coworkers, 1977; Horn and coworkers, 1986; and Thassani and coworkers, 1980) suggests that ethanol itself does not contribute directly to ethanol-induced fibrogenesis. The precise mechanism for ethanol-induced cirrhosis is not yet known. However, the following theories have been proposed:

1. Ethanol induced malnutrition

   Historically, the first mechanism proposed to explain the pathogenesis of ethanol-induced cirrhosis was primary malnutrition caused by the displacement of other nutrients in the diet with ethanol. The studies conducted in human volunteers and primates have shown that both the initial liver lesion,
the fatty liver (Lieber and coworkers, 1965), and the ultimate stage of cirrhosis (Lieber and De Carli, 1974) can be produced by ethanol in the absence of dietary deficiencies and even in the presence of enriched diets (Lieber and Rubin, 1968). Studies conducted by Gruchow and coworkers suggested that alcoholic calories are additive for light drinkers, but in the diet of moderate and heavy drinkers alcoholic calories replace other sources of energy predominantly carbohydrates (Gruchow et al, 1985). With the increase of ethanol intake, the percent of energy derived from proteins, fat and carbohydrates are reduced. The nutritional quality of the diet also declines (Hillers and Massey, 1985; Sherlock, 1984). This may result in vitamin (Gruchow et al, 1985) and mineral deficiency (Hillers and Massey, 1985). However, this concept has been largely replaced by theories that involve metabolic disturbances caused by ethanol oxidation.

2. Microsomal ethanol oxidation system

   Earlier it was thought that the primary pathway for hepatic ethanol oxidation involved cytosolic alcohol dehydrogenase (ADH). Ethanol oxidation to acetaldehyde via the ADH pathway is associated with a reduction of nicotinamide adenine dinucleotide (NAD) to reduced nicotinamide adenine dinucleotide (NADH) which lead to adenosine-5'-triphosphate (ATP) synthesis. As a net result, ADH mediated ethanol oxidation generates an excess of reducing equivalents, as free NADH, because of inability of NADH removal
systems to fully offset its accumulation resulting in an increased NADH/NAD ratio. This change in the NADH/NAD ratio signals changes in liver metabolism which results in several hepatic and metabolic disorders (Lieber et al, 1959; Lieber and Davidson, 1962). In liver microsomes one of the proposed mechanisms involving non-ADH mediated ethanol oxidation was attributed to a hydrogen peroxide dependent reaction which is promoted by contaminating catalase. This oxidative reaction exhibited a substrate specificity for methanol rather than long chain aliphatic alcohols and was exquisitely sensitive to inhibitors of catalase activity such as azide and cyanide (Orme-Johnson and Ziegler, 1965; Ziegler, 1972). The peroximal catalase enzyme is considered to play a minor part in vivo, even though it is capable of ethanol oxidation, because its activity is dependent upon production of hydrogen peroxide, which has been shown to be low under normal circumstances in the liver (Oshino and coworkers, 1975). Lieber and DeCarli (1968,1970) proposed the presence of a system distinct from ADH and catalase and named it the microsomal ethanol oxidizing system (MEOS). According to this pathway, reduced nicotinamide adenine dinucleotide phosphate (NADPH) is utilized (Figure 1). The rates for MEOS-catalyzed ethanol oxidation is relatively high ($K_m = 10$ mM) and the activity is thought to be dependent upon cytochrome P-450 system (Lieber, 1991).
CH$_3$CH$_2$OH + NADPH + H$^+$ + O$_2$ $\rightarrow$ MEOS $\rightarrow$ CH$_3$CHO + NADP$^+$ + 2H$_2$O

Figure 1. Oxidation of ethanol by MEOS.

The new nomenclature for the MEOS is cytochrome P-450IIE1 (Nebert and coworkers, 1987) and this isoenzyme appears to play an important role in upsetting the normal NADP/NADPH balance.

3. Formation of fatty acid ethyl ester

Laposata and Lange (1986) postulated that fatty acid ethyl esters may have a role in the production of ethanol induced injury. They proposed a non-oxidative pathway of ethanol to form fatty acid ethyl esters. Matsuzaki and Lieber (1977) reported that mitochondrial functions, including fatty acid (FA) oxidation, are depressed even in presence of low concentration of acetaldehyde. This may be as a consequence of altered cellular membranes (Waring et al, 1981, 1982). The capacity of ethanol to form ethyl esters in vivo had been demonstrated by Goodman and Deykin (1963) and also by Lange (Lange, 1982; Mongelson and Lange, 1984). It was found that in acutely intoxicated subjects, the concentration of fatty acid ethyl esters was significantly higher than in controls in tissues such as pancreas, liver, heart and adipose tissue (Laposata and Lange, 1986). Since this non-oxidative ethanol metabolism occurs in humans in organs most commonly injured by ethanol
abuse, and since some of these organs lack oxidative ethanol metabolism, this might be a probable mechanism for ethanol induced hepatic injury.

4. Acetaldehyde induced defects in connective tissue metabolism

Another possible mechanism for ethanol induced hepatic injury is the involvement of ethanol or its metabolites on connective tissue metabolism. There are data which suggest that ethanol increases in vitro incorporation of radioactive proline into collagen in liver tissue from patients with cirrhosis (Chen and Leevy, 1975) yet there is no clear evidence that this increase represents the specific effect of ethanol in collagen production. The lack of a stimulatory effect of ethanol on collagen production in cultured liver fibroblasts (Galambos and coworkers, 1977) and lung fibroblasts (Thassani and coworkers, 1980) and baboon liver myofibroblasts (Brenner and Chojkier, 1981) suggests that ethanol itself does not cause ethanol induced hepatic fibrogenesis directly. All known pathways of ethanol oxidation in liver result in production of acetaldehyde, which is further metabolized to acetate (Figure 2). A number of studies have documented the substantial involvement of the metabolites of ethanol oxidation in the manifestation of metabolic and toxic effects of ethanol. Acetaldehyde is the intermediary metabolite formed in the liver during ethanol oxidation (Erickson and Sippel, 1977). Leiber (1982) showed that acetaldehyde mediated many of the biological effects of ethanol. It is widely accepted that more than 90 percent of the acetaldehyde formed is oxidized by the liver (Lindros, 1974).
Holt and coworkers (1984) reported that acetaldehyde stimulated collagen biosynthesis by human fibroblasts \textit{in vitro}.

\[
\text{Ethanol} \rightarrow \text{ADH} \rightarrow \text{Acetaldehyde} \rightarrow \text{ALDH} \rightarrow \text{Acetate}
\]

Figure 2. The stages of the metabolism of ethanol in the liver.

Ethanol metabolism in the liver
ADH = alcohol dehydrogenase
ALDH = acetaldehyde dehydrogenase

It was determined that acetaldehyde, at the concentrations found in the liver during ethyl alcohol abuse (Erikson and Sippel, 1977; Lindros, 1975), stimulated collagen and noncollagen protein production by fetal fibroblasts and human skin and hepatic fibroblasts. Similarly, Savolainen and coworkers (1984) demonstrated that acetaldehyde stimulated collagen production in baboon liver myofibroblasts. Brenner and Choijkier (1987) also showed that the acetaldehyde increases collagen gene transcription in cultured human fibroblasts and increases collagen mRNA level.
However, Moshage and coworkers (1990) noted that fat-storing cells (FSC, also called perisinusoidal lipocytes or Ito cells), the principal cells residing in the Disse space of the liver, are likely effector cells in increased production of type I collagen in alcoholic liver fibrosis. FSC’s can transform into activated transitional cells during ethanol-induced liver injury in experimental animals (Mak and coworkers, 1984, 1988) and in humans (Horn and coworkers, 1986). Various researchers (Brenner and Chojkier, 1987; Erickson and Sippel, 1977; Galambos et al, 1977; and Lieber, 1982) have also demonstrated that upon exposure to acetaldehyde, FSC’s can synthesize and release different types of collagen, both in vivo or in vitro.

5. Generation of free radicals and role of antioxidants

Another possible mechanism of cellular injury contributing to acute ethanol-induced liver disease is the generation of free radicals or partially reduced oxygen species (PROS). This hypothesis was first proposed by William and Barry (1987). It is based on the observation that hepatocyte membranes altered by acetaldehyde stimulated neutrophils produced superoxide anions.

Chemistry and Cytotoxicity of Reactive Oxygen Metabolites

It is paradoxical that although oxygen (O₂) is essential for aerobic life forms, too much O₂ or its inappropriate metabolism can lead to toxicity in an organism. In most organisms, the majority of energy is derived by controlled,
four-electron reduction of $O_2$ to form water ($H_2O$) by the mitochondrial electron transport system in the form of cellular ATP (Grisham and McCord, 1986). During the course of normal metabolism, however, $O_2$ can accept less than four electrons to form reactive $O_2$ metabolites that may be cytotoxic to cells. Molecular $O_2$ is not very fast to react because of spin restriction. Molecular $O_2$ exist in a triplet or diradical state, whereas most organic compound are in the singlet state. For $O_2$ to react with compounds in the singlet state, the spin of one of its outermost electrons must be reversed. In the absence of a catalyst, this spin reversal is much slower than the collision frequency. Spin restriction can be overcome if enough energy for activation is available to excite $O_2$, causing spin inversion or pairing of the outer electrons, thus producing singlet $O_2^\cdot$, a potent oxidant with an extremely short life time (Grisham and McCord, 1986) (Figure 3).

\begin{align*}
a. & \quad O_2 + e^- \rightarrow O_2^\cdot \\
b. & \quad O_2 + 2e^- + 2H^+ \rightarrow H_2O_2 \\
c. & \quad O_2^\cdot + H_2O_2 + H^+ \rightarrow O_2 + H_2O + \cdot OH
\end{align*}

**Figure 3.** Formation of PROS. Reactions indicate the formation of: a) superoxide radicals, b) hydrogen peroxide and c) hydroxyl radical.
Some investigators have shown that \( \text{O}_2^- \) may exert cytotoxicity by inactivation of enzymes essential to cells including transfer ribonuclease (tRNase), and glyceraldehyde-3-phosphate dehydrogenase (Kellogg and Fridovich, 1977; Lavalle et al, 1973).

Although \( \text{O}_2 \) can not be directly reduced by three electrons; \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) can interact in the presence of certain transition metals, metal chelates, or hemoproteins to yield potent oxidizing agents such as hydroxyl free radicals (\(^\cdot\text{OH}\)).

\[
a. \quad \text{O}_2^- + \text{Fe}^{3+} \rightarrow \text{O}_2 + \text{Fe}^{2+} \\
b. \quad \text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow ^\cdot\text{OH} + \text{OH}^- + \text{Fe}^{3+} \\
c. \quad \text{O}_2^- + \text{H}_2\text{O}_2 \rightarrow ^\cdot\text{OH} + \text{OH}^- + \text{O}_2
\]

Figure 4. Generation of hydroxyl free radical by Fenton reaction. Reaction c represents the sum of reaction a and b.

The reactions depicted in Figure 4 have been called the iron-catalyzed Haaber-Weiss reaction or \( \text{O}_2^- \) driven Fenton reaction. Naturally occurring iron containing compounds active in catalyzing \(^\cdot\text{OH}\) formation include transferrin (Motohashi and Mori, 1983), ADP-Fe\(^{3+}\) (Tien and Aust, 1982), hemoglobin (Sadrzadeh et al, 1984) and ferritin (Thomas et al, 1985). The \( \text{O}_2^- \) provides
the electron to reduce ferric iron to ferrous iron, which in turn reduces $H_2O_2$ to yield $\cdot OH$. Since $O_2^-\cdot$ formation is always accompanied by the production of $H_2O_2$, metal-catalyzed formation of $\cdot OH$ is a possibility. The $\cdot OH$ is the most reactive free radical produced in biological systems (Grisham and McCord, 1986). The hydroxyl free radical and other similar compounds are the only metabolites formed in the biological systems that can remove methylene hydrogen atoms from polyunsaturated fatty acids and thus initiate lipid peroxidation (Figure 5) (Grisham and McCord, 1986).

$$ROH + \cdot OH \rightarrow H_2O + R^\cdot$$
$$R^\cdot + O_2 \rightarrow ROO^\cdot$$
$$ROO^\cdot + RH \rightarrow ROOH + R^\cdot$$
$$R^\cdot + R^\cdot \rightarrow R-R$$

Fig. 5 Initiation of lipid peroxidation. RH, R^\cdot, ROO^\cdot and ROOH represents polyunsaturated lipid, lipid alkyl radical, lipid perhydroxyl radical and lipid hydroperoxide, respectively.

Oxidative free radicals are formed \textit{in vivo} from molecular oxygen. Detoxification of these reactive species is a prerequisite of aerobic life. Multiple lines of defense against pro-oxidant species have evolved in biological systems. However, such defenses can be overcome by free radicals and cause cellular
injury especially when produced in excessive amounts. Thus, the disturbed balance in favor of pro-oxidative species has been denoted as oxidative stress.

Development of fatty liver induced by the administration of a large single doses of ethanol can be attenuated by administering antioxidants (Di Luzio, 1964). It could therefore be suggested that ethanol produces free radical disturbances in the liver that result in an enhanced membrane lipid peroxidation. The mechanism responsible for hepatic lipid peroxidation during ethanol intoxication remain controversial. Because of its high reactivity, the \( \cdot \text{OH} \) radical is the most likely candidate having a cytotoxic role (Slater, 1972). Cederbaum (1987) showed that microsomal \( \cdot \text{OH} \) generation is significantly increased after chronic ethanol consumption. This increase is probably related to enhanced \( \text{O}_2^- \) radical production which can occur at the site of either NADPH-cytochrome P450 reductase (Cederbaum, 1987) or cytochrome P450 itself (Ekstrom and Ingelman-Sundberg, 1986). Other sources of free radical generation may be ethanol itself (Slater, 1972), iron-oxygen complexes (Minotti and Aust, 1987) and xanthine oxidase (Oei et al, 1986; Sultatos, 1988).

The main pathway for ethanol metabolism involves hepatic ADH, a cytosolic enzyme to catalyze the conversion of ethanol to acetaldehyde. Hydrogen is transferred from ethanol to the cofactor NAD which is converted to its reduced form NADH (Lieber, 1991). The acetaldehyde produced again loses hydrogen and is converted to acetate most of which is released into the bloodstream.
The resulting enhanced NADH/NAD ratio, produce a change in the ratio of those metabolites that are dependent on the NADH-NAD couple for reduction. Ethanol induced ketosis and enhanced purine breakdown (Faller and Fox, 1982) causes increased production of PROS by xanthine oxidase. This is supported by the protective effects of allopurinol against ethanol-induced lipid peroxidation (Kato et al, 1990).

It has been well established that alpha-tocopherol (vitamin E) represents a last line of defense against lipid peroxidation by scavenging the ROO* radicals (Wittig, 1980; Burton et al, 1983). There is documented literature that other weak natural antioxidants are also capable of inhibiting ethanol-induced liver damage.

Natural Antioxidants

There are at least four nutrients which, theoretically, could have significant antioxidant effects: mono-unsaturated fatty acids, ascorbic acid (vitamin C), alpha-tocopherol (vitamin E), and beta-carotene.

It has been documented that cysteine, ascorbic acid and vitamin E afford a protective effect against ethanol induced toxicity. All the above mentioned compounds are antioxidants, suggesting the involvement of an oxidative mechanism. Houglum and coworkers (1991) reported that vitamin E inhibits collagen gene expression in cultured human fibroblasts mediated through induction of lipid peroxidation. This effect was also observed with methylene
blue which is also a scavenger of free radicals.

**Probucol**

Probucol, 4,4’ (isopropylidenedithio) bis (2,6-di-t-butyl-phenol) is an antilipidemic compound in current clinical use. Probucol has been characterized as a potent antioxidant. It inhibits atherogenesis by inhibiting oxidative low density lipoprotein (LDL) modification. The sulfo-phenolic structure of probucol (Fig. 6) suggests that it is an oxygen free radical scavenger.

Probucol is poorly absorbed from the gastrointestinal tract; only 2-8% of the peroral dose reaches the circulation (Marshall, 1982). The peak plasma concentration usually occurs within 2 hour of administration. Heeg and coworkers (1984) reported 6% absorption of administered probucol in adult male Sprague-Dawley rats.

A majority of the absorbed dose (72%) is found as probucol in the liver (Heeg et al, 1984). The concentration in the liver is 130-200 times the concentration in the plasma. The oral studies indicate that most of an absorbed dose of probucol is excreted primarily in the feces. Elimination of most of the labeled drug in bile duct-cannulated rats (Table 5) suggests that most of the absorbed drug is secreted in the bile (Heeg et al, 1984). Also, due to its lipophilicity and relatively long half life, probucol is accumulated in the fat with repeated oral regimens of probucol. Probucol is poorly absorbed from the gastrointestinal tract, with only 2-8% of the dose reaching the circulation.
(Marshall FN, 1982). Probucol is transported from the gastrointestinal tract within chylomicron and VLDL. With continuous oral administration of probucol, the blood levels rise and stay relatively constant after a period of approximately 3 months (Taylor et al, 1978). Probucol accumulates in the adipose tissue, and may persist there and in the circulation for more than 6 months (Taylor et al, 1978). Since probucol is a very fat soluble drug and leads to a depot effect, differences in eating times do not produce any significant fluctuation in plasma levels or hepatic tissue levels of the drug.
Figure 6. Structure of Probucol
Table 5. The cumulative percent of the radioactive dose excreted in the urine and feces of male rats and biliary excretion in bile duct-cannulated male rats^a.  

<table>
<thead>
<tr>
<th>Oral</th>
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<tr>
<td>Day</td>
<td>Feces</td>
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<td>1</td>
<td>0.09 ± 0.03</td>
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<tr>
<td>2</td>
<td>0.19 ± 0.07</td>
<td>92.12 ± 1.26</td>
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<tr>
<td>3</td>
<td>0.23 ± 0.08</td>
<td>92.82 ± 1.12</td>
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<tr>
<td>7</td>
<td>0.28 ± 0.09</td>
<td>93.60 ± 0.96</td>
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</table>

^a A 10-mg/kg [\(^{14}\text{C}\)] probucol dose. Data expressed as mean ± SD; n = 4 for urine and feces data; n = 5-6 for bile data (modified from Heeg et al, 1984).  
^b data not available.
To further investigate this relationship between ethanol consumption, oxidative stress, and hepatic injury; this project tested the following specific aims:

Specific aims:

It is the objective of this research project to investigate the hypothesis that the probable mechanism of ethanol-induced hepatic injury results from the generation of oxygen free radicals.

To test this hypothesis, a controlled study was performed in which probucol, a potent antioxidant, was administered concurrently to ethanol-treated and non ethanol-treated rats. The following experiments which were designed to measure the amount of total liver collagen, and examine other connective tissue changes between the treatment groups, were performed on the livers of each rat:

I. Measurement of lipid levels and estimation of the extent of lipid peroxidation in the liver.

II. Measurement of total collagen and acid-pepsin insoluble collagen in the liver.

III. Determination of the relative amounts of early and advanced glycosylation end products in the liver connective tissue matrix.
MATERIALS & METHODS

Animals and Housing:

Male Sprague Dawley rats weighing 250-300 g, obtained from Bantin & Kingman Inc, (Fremont, CA), were used in this study. The rats were housed in individual wire-mesh hanging cages over wood chip bedding. The Rat Chow^TM Animal Diet 5012 obtained from Purina Mills Inc, (St Louis, MO) and liquid provided were available ad lib during 12 hour light-dark cycles with lights on at 7 A.M. All animals were allowed a minimum of 7 days acclimatization before starting the experiments.

Reagents & Chemicals:

Reagents and chemicals used were: Chloramine T (N-chloro p-toluene sulfonamide sodium salt), collagenase Type IV Lot 122H681, calcium cyclamate, ethylene glycol monomethyl ether, fluorescamine, proteinase K Lot 31H68362, pronase E Lot 112H0299, pepsin A Lot 70H8225, probucol, 2-thiobarbituric acid (4,6 dihydroxypyrimidine 2-thiol), p-dimethylamino benzaldehyde(Ehrlich reagent), 1,1,3,3 tetramethoxypropane, thymolphthalein (5'-5" Diisopropyl-2'-2" dimethylphenolphthalein) and Trizma HCl (Tris[hydroxymethyl]-amino methane HCl). They were obtained from Sigma Chemical Company, (St Louis, MO 63178). Acetonitrile, chloroform, ethyl
ether, methanol and trichloroacetic acid were obtained from Fisher Scientific Inc, (Fairlawn, NJ 07410).

EXPERIMENTAL PROTOCOL

Twenty eight male Sprague Dawley rats were divided randomly into four groups consisting of:
1. a control group,
2. an ethanol-treated group,
3. a probucol-ethanol-treated group, and
4. a probucol-treated control group

All ethanol treated rats were given a 19% v/v ethanol solution mixed with cyclamic acid hemicalcium salt as sweetener whereas the other animals were given the sweetened water without ethanol. The rats were able to access the solution ad lib. It was observed that rats dosed with 19% v/v ethanolic solution consumed 27.5 ± 2.5 ml of fluid over a 24 hour period while the rats dosed with sweetened water without ethanol consumed 35.0 ± 5.0 ml of fluid.

The ethyl alcoholic solution was prepared according to the following formula:
Ethanol (95%)................ 200 ml
Calcium Cyclamate........... 1.0 g
Water, qs to............... 1000 ml

This treatment was carried out for the duration of 5 months. Bankowski and Pawlicka (Bankowski et al, 1987) were able to produce collagen content changes in livers of chronically intoxicated rats with 10 percent (v/v) ethanol versus controls. In this study, the rats were dosed at 19 percent (v/v) ethanol (1.9 times the above study) to ensure significant liver matrix changes.

Dosing of rats with Probucol

Probucol-ethanol treated rat group and probucol control rat group were dosed with probucol via the rat chow. A solution was made by dissolving 1.75 grams of probucol in 180 ml of ethyl ether. Fifteen hundred grams of rat chow were then soaked in the above solution and the ether was allowed to evaporate. The concentration of probucol in the prepared food was approximately 120 mg probucol per 100 g of food.

It was found, in preliminary studies conducted in this laboratory, that the rats in the control group and the probucol group (groups 1 and 4) consumed $26.8 \pm 1.05$ grams of food/24 hr while the ethanol treated group (groups 2 and 3) consumed $20.1 \pm 0.42$ grams of food/24 hr respectively. This finding
gave us a dose of 58 mg/kg body weight (which is one half of the cholesterol lowering dose in humans on a surface area basis).

Animal preparation

At the end of 5 months of treatment, the rats were anesthetized with ethyl ether, and blood was withdrawn by intracardiac puncture. Then the rats were sacrificed and the livers were isolated rapidly. The livers were then washed with distilled water, weighed and rapidly frozen by placing them on dry ice. A section of some of the livers were fixed in buffered formalin for pathological evaluation by a veterinary pathologist. The frozen livers were then lyophilized in a Thermovac freeze drier for 72 hours. The lyophilized livers were weighed, powdered in a blender and were stored at -20 degree C. The following analytical tests were performed on the lyophilized livers.

Total Protein and Hydroxyproline analysis

The lyophilized liver sample from each rat (0.05 gm) was used for determination of total hydroxyproline content (Stagemann H & Stalder K, 1967 and Woesnner J Jr, 1961). The lyophilized sample was hydrolyzed by addition of 2 ml 6 N HCl at 107 degree C for 16 hr using Thelco Model 18 oven (GCA/Precision Scientific Inc, Chicago, IL 60647). After the incubation, the sample was neutralized by dropwise addition of 2.5 N NaOH solution using thymolphthalein as the indicator. One ml of the above neutralized solution was
then pipetted into 16 x 150 cm test tubes and to it was added 1 ml of pH 6 citrate-phosphate buffer. To this solution, 1 ml of chloramine T was added to each test tube. The test tube contents were mixed and allowed to stand for 20 minutes at room temperature. The chloramine T was then destroyed by adding 1 ml 3.15 M perchloric acid (prepared by diluting 27.0 ml of 70 % perchloric acid to 100 ml). The contents were mixed and allowed to stand for 5 minutes. Finally 1 ml of 20 % p-dimethylamino-benzaldehyde solution was added and the mixture was shaken until no schlieren can be seen. The tubes are placed in a 60 degree C waterbath for 20 minutes. The developed color is stable for at least 1 hr. The absorbance of the solution is determined spectrophotometrically using a Gilford Stasar III spectrophotometer (Gilford Instrument Laboratories Inc, Oberlin, OH 44074) at 557 nm. Standards were concurrently run with the assay and a standard curve developed. This assay provides information about development of ethanol-induced cirrhosis. The hydroxyproline content assay is used to measure the amount of collagen. Since the collagen is the major protein which possesses high quantities of hydroxyproline, its determination can accurately estimate the amount of collagen.

Early glycated protein levels

Rosenberg and coworkers (Rosenberg et al, 1979) devised a method for the determination of early glycated protein products (EGP's). Briefly, according to this method, a portion (0.10 g) of the lyophilized liver powder was
autoclaved for 18 hours at 110 degree C in distilled water. After cooling the autoclaved mixture, 1 ml of 1 N oxalic acid was then added and heated again at 100 degree C for 1 hour. This was followed by addition of 1 ml of 40% trichloroacetic acid. The samples were then centrifuged at 10,000 x g using IEC B 20A highspeed centrifuge (Damon/IEC Division Inc, Needham Heights, MA 02194) to remove the debris. From the resulting supernatant, 1 ml of supernatant was diluted with 1 ml distilled water and to it was added 1 ml of 2-thiobarbituric acid (TBA) [prepared by dissolving 0.05 mole of TBA per liter in distilled water at 60 degree C]. The cooled TBA solution was then added to the supernatant and incubated at 40 degree C for another 30 minutes. After cooling to room temperature, the solution was analyzed at 443 nm using the Gilford Stasar III spectrophotometer. The color change developed due to the reaction of 5-HMF (5-Hydroxymethyl furfural) with TBA. The concentrations of the test solutions were calculated from standard curves.

Early glycated collagen products have been shown to change the normal interactions between collagen molecules and other macromolecules in the extracellular matrix.

Advanced Glycosylation End Product (AGEP) analysis

The methods of MacDonald and coworkers (1992) was used for determining the relative amounts of advanced glycated products (AGP's). According to the procedure, portions of the lyophilized liver samples (0.05 g)
were suspended in Clostridium histolyticum collagenase at a concentration of 250 IU/ml. These digest solutions were placed in 37 degree C waterbath for 24 hours. After 24 hours, Proteinase K at the concentration of 250 IU/ml was added to the sample and the samples were again placed in a 37 degree C waterbath for 24 hours. After 24 hours, the digested samples were centrifuged at 10,000 x g using the IEC B 20A highspeed centrifuge and the supernatants were measured for fluorescence, to determine the relative amounts of AGEP’s. Fluorescence analysis was done on a J4-8961 Aminco-Boman spectrophotofluorometer (American Instrument Co, Silver spring, MD 20910) with excitation and emission wavelengths set at 370 nm and 440 nm, respectively.

AGP is an index of the amount of irreversibly crosslinked collagen present in the body. It represents connective tissue changes and is dependent upon the age and changes resulting from several diseased states.

**Acid-pepsin soluble and insoluble collagen levels**

A portion of the lyophilized liver samples were tested for acid pepsin solubility similarly to the method described by Brownlee and coworkers (1988). Distilled water was added to 0.22 g of the lyophilized samples and the pH of the sample adjusted to 2.5 with formic acid. Pepsin was then added in the ratio of 1:200 to a final volume of 30 ml. This mixture was shaken gently for 72 hours at 37 degree C using Lab-Tek Mixer/shaker (Ames-Lab-Tek Inc,
Westmont, IL). After incubation, a 2 ml sample of the digest was taken for determination of total hydroxyproline and total protein content using the methods described above. The rest of the digest was centrifuged at 18,000 x g for 1 hour using an IEC-20 A high speed centrifuge. The supernatant, which contains the acid soluble content was also analyzed for total protein and hydroxyproline content using the methods of Bradford (Bradford M, 1976), Stagemann and Stalder (Stagemann H & Stalder K, 1967) and Woesnner Jr (Woesnner J Jr, 1961). The precipitated, nondigested products after centrifugation were lyophilized, weighed and then analyzed for total protein and hydroxyproline content. Appropriate controls containing pepsin solution of identical concentration and adjusted to the same pH were also run concurrently. The amount of hydroxyproline in non digested liver tissue would represent acid-pepsin-insoluble collagen. An increase in acid pepsin insoluble collagen suggests divergence from normal matrix interactions and may represent increased covalent crosslinks in the tissue matrix.

Measurement of lipid content:

Lipid content was measured gravimetrically by weighing 1 gram of freeze-dried liver (accurate to 0.001 g), extracting the lipid soluble fraction with chloroform-methanol (2:1), then reweighing the freeze-dried liver after filtration and drying.
Measurement of Lipid Peroxidation

The Snell and Mullock (Snell K & Mullock B, 1987) method for the measurement of lipid peroxides was used in this study. According to the method, 0.05 g of the sample is weighed and 1 ml of 10 M phosphate buffer and 1 ml of 20% w/v trichloroacetic acid (TCAA) and 2 ml of 0.67% w/v TBA was added and mixed gently. The solution was heated in a lightly stoppered tube at 100 degree C in a boiling water bath for 10 minutes. The colored product was extracted with 5 ml of 5-Butanol and the absorbance was read at 515 nm excitation and 555 nm emission using J4-8961 Aminco-Boman spectrofluorometer. The standard was made by using 1,1,3,3-tetramethoxypropane which was converted to malondialdehyde (MDA) during the reaction. The method determines the amount of aldehydes formed by degradation of hydroperoxide, including MDA which is used as a standard.

Pathological evaluation

Some of the livers were evaluated for pathological changes and fat deposition. After removal, a section of liver was immersed and fixed in 10% buffered formalin. The pathological examination and staining was done by a qualified veterinary pathologist. These procedures were done to observe any pathological dissimilarities between the treatment groups.
Units

The EGP data is expressed in relationship to mg protein and mcg hydroxyproline. The advanced glycosylated product (AGP) formation is expressed as fluorescence units/g freeze dried liver. The amount of collagenase-protease K enzyme insoluble and soluble collagen is expressed as mg hydroxyproline/g freeze dried liver present in acid pepsin nondigested collagen. The amount of lipid peroxidation is expressed as mg lipid peroxidation/g freeze dried liver.

Data Interpretation

Since the rat data were evaluated individually, statistical analysis between the treatment groups was possible. Statistical analysis was done using SPSS for Windows [Student Version (release 6.0.1; Oct 21, 1993)] software. Statistical analysis was performed between the treatment groups using the pooled t-test for all of the above mentioned tests, except the pathological evaluation, in order to test the hypothesis. The level of significance of p < 0.05 was accepted to be statistically different.
RESULTS

The amount of lipid peroxidation in the liver was significantly higher in control and ethanol-treated rats when compared to either of the probucol treated groups (Tables 7 and 12). The difference in the amount of lipid peroxidation in chronically ethanol-treated rats was highly significant when compared to the probucol treated groups (Figure 7). The probucol treated and probucol-ethanol-treated rats had similar amounts of lipid peroxidation, but less, in each, than that observed in the ethanol-treated and control groups.

Lipid content was 16.9% higher in the ethanol-treated group when compared to controls (Table 6).

The mean total hydroxyproline content of the livers from all the treatment groups are shown in Table 8. The total hydroxyproline content in the case of chronically ethanol-treated rats was significantly higher when compared to all other treatment groups (control, probucol-treated, and probucol-ethanol-treated rats) (Table 12). The hydroxyproline (HP) level of the control group was similar to that of probucol-treated rats, with the probucol-ethanol-treated group being slightly lower than the two. However, the differences among the three groups (controls, probucol-treated and probucol-ethanol-treated rats) were not significant (Figure 8). There were no significant differences between the control group and the probucol-treated groups.
The AGP's, expressed as fluorescence units per gram of freeze dried liver, was highest in control rats (Tables 9 and 14). All of the four treatment groups were significantly different from one another with the probucol-treated groups showing the lowest AGP levels (Figure 9).

The comparison of mean collagenase-protease K enzyme soluble hydroxyproline (HP) levels in the freeze dried livers of all the treatment groups is given in Table 10. The HP levels for enzyme soluble collagen was similar among controls, probucol-treated and probucol-ethanol treated rats. The HP level in chronically ethanol-treated rats was lower than that of any of the three groups versus controls, probucol-treated and probucol-ethanol treated rats (Table 15). The differences when compared the each of the three groups was found to be highly significant (Figure 10).

The amounts of insoluble HP in each of the treatment group were obtained by subtracting the enzyme soluble HP levels from the total HP levels of each sample (n). The results were then expressed as mg of HP (insoluble) per g of freeze dried liver and are enumerated in Table 11. The amount of enzyme insoluble HP was found to be highest in the ethanol-treated rats while lower but similar amounts were found in other three groups (Figure 11). The difference observed in the ethanol-treated rats was highly significant when compared to controls, probucol-treated and probucol-ethanol-treated rats (Table 16).

Pathological evaluation carried out by a certified veterinary pathologist was inconclusive. The only difference noted between the treatment groups was
the greater amount of necrosis in the ethanol-treated group. The probucol treatment groups had slightly greater vacuolization.
Table 6. Comparison of mean lipid levels in the freeze dried livers of all treatment groups.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>MEAN (g Lipid/g FDL)</th>
<th>95 % CONF. INT.</th>
<th>SE of MEAN</th>
</tr>
</thead>
<tbody>
<tr>
<td>C (n = 7)</td>
<td>0.183</td>
<td>0.171, 0.195</td>
<td>0.006</td>
</tr>
<tr>
<td>A (n = 7)</td>
<td>0.214</td>
<td>0.196, 0.232</td>
<td>0.009</td>
</tr>
<tr>
<td>P (n = 7)</td>
<td>0.207</td>
<td>0.191, 0.223</td>
<td>0.008</td>
</tr>
<tr>
<td>PA (n = 7)</td>
<td>0.226</td>
<td>0.212, 0.240</td>
<td>0.007</td>
</tr>
</tbody>
</table>

Data are presented as Mean ± SE and expressed in g of lipid per gram of freeze-dried liver. Confidence interval is abbreviated as Conf. Int.

C = Control; A = Ethanol; P = Probucol-treated; PA = Probucol-ethanol-treated.
Table 7. Comparison of mean lipid peroxidation levels in the freeze dried livers of all treatment groups.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>MEAN (mg LP/g FDL)</th>
<th>95 % CONF. INT.</th>
<th>SE of MEAN</th>
</tr>
</thead>
<tbody>
<tr>
<td>C (n = 7)</td>
<td>1.40</td>
<td>1.08, 1.72</td>
<td>0.13</td>
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<tr>
<td>A (n = 7)</td>
<td>1.31</td>
<td>1.05, 1.57</td>
<td>0.11</td>
</tr>
<tr>
<td>P (n = 7)</td>
<td>0.24*</td>
<td>0.0001, 0.41</td>
<td>0.09</td>
</tr>
<tr>
<td>PA (n = 7)</td>
<td>0.21*</td>
<td>0.03, 0.46</td>
<td>0.08</td>
</tr>
</tbody>
</table>

Data are presented as Mean ± SE and expressed in mg of lipid peroxidation per gram of freeze-dried liver. Confidence interval is abbreviated as Conf. Int.

C = Control; A = Ethanol; P = Probucol-treated; PA = Probucol-ethanol-treated.

* denotes values statistically significant (p < 0.05) compared to control and ethanol-treated groups.
Figure 7. Lipid peroxidation values expressed as mg lipid peroxidation/g freeze-dried livers. C = Control, A = Ethanol, P = Probucol-treated, PA = Probucol-ethanol-treated. The standard error bars indicate the spread of standard error of mean (SEM).

* denotes significance (p < 0.05) compared to control and ethanol treated rats.
Lipid Peroxidation Levels

![Graph showing lipid peroxidation levels across different treatment groups. The x-axis represents treatment groups (C, A, P, PA) and the y-axis represents mg LP/g FD Liver. The bars indicate the levels with error bars, and significance markers (*).]
Table 8. Comparison of mean hydroxyproline levels in the freeze dried livers of all treatment groups.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>MEAN mg HP/g FDL</th>
<th>95 % CONF. INT.</th>
<th>SE of MEAN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
</tr>
<tr>
<td>C</td>
<td>1.13</td>
<td>0.91, 1.35</td>
<td>0.08</td>
</tr>
<tr>
<td>(n = 6)</td>
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<tr>
<td>A</td>
<td>1.66*</td>
<td>1.51, 1.79</td>
<td>0.05</td>
</tr>
<tr>
<td>(n = 6)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>P</td>
<td>1.15</td>
<td>1.02, 1.27</td>
<td>0.05</td>
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<tr>
<td>(n = 6)</td>
<td></td>
<td></td>
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<tr>
<td>PA</td>
<td>1.04</td>
<td>0.95, 1.12</td>
<td>0.03</td>
</tr>
<tr>
<td>(n = 6)</td>
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</tbody>
</table>

Data is presented as Mean ± SE and expressed in mg Hydroxyproline/g of freeze dried liver. Confidence interval is abbreviated as Conf. Int.

C = Control; A = Ethanol; P = Probucol-treated; PA = Probucol-ethanol-treated.

* denotes values statistically significant (p<0.05) compared to all other treatment groups.
Figure 8. Total hydroxyproline values expressed as mg Hydroxyproline/g freeze dried livers. C = Control, A = Ethanol, P = Probucol-treated, PA = Probucol-ethanol-treated. The standard error bars indicate the spread of SEM.

* denotes significance (p < 0.05) compared to control, probucol and probucol-ethanol-treated rats.
Hydroxyproline (HP) levels

![Bar graph showing HP levels for different treatment groups.]  

**mg HP / g FDL**

- **Treatment Group**
  - C
  - A
  - P
  - PA

- **Shaded area** indicates mg HP / g FDL for each treatment group.
TABLE 9. Comparison of mean advanced glycosylated products (AGP's) levels in the freeze dried livers of all treatment groups.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>MEAN</th>
<th>95 % CONF. INT.</th>
<th>SE of MEAN</th>
</tr>
</thead>
<tbody>
<tr>
<td>FU / g FDL</td>
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<td></td>
</tr>
<tr>
<td>C (n = 6)</td>
<td>1697</td>
<td>1515, 1882</td>
<td>66.1</td>
</tr>
<tr>
<td>A (n = 6)</td>
<td>1356&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1223, 1488</td>
<td>47.8</td>
</tr>
<tr>
<td>P (n = 6)</td>
<td>1167&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1130, 1203</td>
<td>13.2</td>
</tr>
<tr>
<td>PA (n = 6)</td>
<td>958&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>928, 989</td>
<td>10.9</td>
</tr>
</tbody>
</table>

Data is presented as Mean ± SE and expressed in fluorescence units / g of freeze dried liver. Confidence interval is abbreviated as Confi. Int.

C = Control; A = Ethanol; P = Probucol-treated; PA = Probucol-ethanol-treated.

<sup>abc</sup> denotes values statistically significant (p<0.05) compared to <sup>a</sup> controls, <sup>b</sup> ethanol-treated, and <sup>c</sup> probucol-treated rats.
Figure 9. Advanced glycated products (AGP's) values expressed as fluorescence units per gram freeze dried livers. C = Control, A = Ethanol, P = Probucol-treated, PA = Probucol-ethanol-treated. The standard error bars indicate the spread of SEM.

PA - * denotes significance (p < 0.05) when compared to control, ethanol-treated, and probucol-treated group.

P - * denotes significance (p < 0.05) when compared to control, ethanol-treated, and probucol-ethanol-treated group.

A - * denotes significance (p < 0.05) when compared to control group.
**Advanced Glycated Product**

![Bar chart showing FU (units) per gram of FDL liver for different treatment groups: C, A, P, PA. The chart indicates statistically significant differences marked with asterisks (*).](image-url)
Table 10. Comparison of mean collagenase-protease K enzyme soluble hydroxyproline levels in the freeze dried livers of all treatment groups.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>MEAN mg HP / g FDL</th>
<th>95 % CONF. INT.</th>
<th>SE of MEAN</th>
</tr>
</thead>
<tbody>
<tr>
<td>C (n = 5)</td>
<td>463</td>
<td>429, 496</td>
<td>12.1</td>
</tr>
<tr>
<td>A (n = 5)</td>
<td>321&lt;sup&gt;a&lt;/sup&gt;</td>
<td>311, 330</td>
<td>3.39</td>
</tr>
<tr>
<td>P (n = 5)</td>
<td>456</td>
<td>374, 539</td>
<td>29.7</td>
</tr>
<tr>
<td>PA (n = 5)</td>
<td>446&lt;sup&gt;b&lt;/sup&gt;</td>
<td>397, 495</td>
<td>17.7</td>
</tr>
</tbody>
</table>

Data is presented as Mean ± SE and expressed in mg hydroxyproline/g of freeze dried liver. C = Control; A = Ethanol; P = Probucol-treated; PA = Probucol-ethanol-treated. Confidence interval is abbreviated as Confl. Int.

<sup>ab</sup> denotes values statistically significant (p < 0.05) compared to <sup>a</sup>controls and/or <sup>b</sup>ethanol-treated rats.
Figure 10. Collagenase-protease K enzyme soluble hydroxyproline values expressed as mg hydroxyproline/g freeze dried livers. C = Control, A = Ethanol, P = Probucol-treated, PA = Probucol-ethanol-treated. The standard error bars indicate the spread of SEM.

* denotes significance (p < 0.05) compared to control, probucol-treated and probucol-ethanol-treated group.
Enzyme soluble HP levels

mg HP / g FDL Liver

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>C</th>
<th>A</th>
<th>P</th>
<th>PA</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>mg HP/ g FDL</em></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

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Table 11. Comparison of mean collagenase-protease K enzyme insoluble hydroxyproline levels in the freeze dried livers of all treatment groups.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>MEAN mg HP / g FDL</th>
<th>95 % CONFI. INT.</th>
<th>SE of MEAN</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>703</td>
<td>439, 968</td>
<td>95.3</td>
</tr>
<tr>
<td>(n = 5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>1382(^a)</td>
<td>1288, 1475</td>
<td>33.7</td>
</tr>
<tr>
<td>(n = 5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>691(^b)</td>
<td>530, 852</td>
<td>57.9</td>
</tr>
<tr>
<td>(n = 5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA</td>
<td>567(^b)</td>
<td>463, 672</td>
<td>37.5</td>
</tr>
<tr>
<td>(n = 5)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data is presented as Mean ± SE and expressed in mg of hydroxyproline/g of freeze dried liver. C = Control; A = Ethanol; P = Probucol-treated; PA = Probucol-ethanol-treated. Confidence interval is abbreviated as Confi. Int.

\(^a\)\(^b\) denotes values statistically significant (p<0.05) compared to \(^a\)controls and \(^b\)ethanol-treated rats.
Figure 11. Collagenase-protease K enzyme insoluble hydroxyproline values expressed as mg hydroxyproline/g freeze dried livers.

C = Control, A = Ethanol, P = Probucol-treated, PA = Probucol-ethanol-treated. The standard error bars indicate the spread of SEM.

* denotes significance (p < 0.05) compared to control, probucol-treated and probucol-ethanol-treated group.
Insoluble HP levels

mg HP / g FDL Liver

Treatment Group

C  A  P  PA

mg HP/ g FDL

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DISCUSSION

Lipid peroxidation

Lipid peroxidation is a free-radical mediated chain reaction which is enhanced as a result of oxidative stress and results in deterioration of polyunsaturated fatty acids. Biomembranes contain substantial amounts of polyunsaturated fatty acids and thus are highly susceptible to peroxidative damage. The increased lipid peroxidation in the liver of ethanol treated rats may be due to intermediate compounds (for example, high molecular weight aldehydes produced and released into blood) after free radical attack on polyunsaturated fatty acids in hepatic biomembrane during metabolism of ethanol (Lung et al, 1990). In this study, it was observed that a large amount of peroxidation occurs normally in controls and ethanol-treated rats. As shown in the data in Table 7 and Figure 7, the amount of lipid peroxidation was reduced by 83% and 85% in the rats treated with probucol and probucol-ethanol-treated rats respectively, suggesting that probucol, by the virtue of its antioxidant properties, was able to reduce the amount of lipid peroxides formed. Many studies have reported significant increase in lipid peroxidation in ethanol-treated rats over controls (Odeleye OE et al, 1992), although our study failed to show any significant differences per gram liver. The lack of difference between the control and ethanol-treated rats in our study (Figure 7) was probably due to the 16.9% increase in the liver-lipid content of the ethanol-
treated rats (Table 6). This would have the tendency to dilute lipid peroxide levels per gram liver. Taking into account the diluting effect, the lipid peroxides appeared to increase about 10% in the ethanol treated rats. Interestingly, probucol was not able to reduce the enhanced lipid levels in the ethanol treated rats. This is probably because acetaldehyde stimulates the production of fatty liver (Lieber, 1991) and probucol does not affect acetaldehyde levels.

A similar decrease in lipid peroxidation, but to lesser extent, has been observed by other researchers in animals treated with Vitamin E (Odeleye OE et al, 1992), thereby suggesting probucol to be a more potent antioxidant than Vitamin E in preventing lipid peroxidation. Similar observations on probucol's antioxidant capabilities have also been made by Minakami and coworkers (1989). Probucol treatment in rats exposed to ethanol did lower the cumulative products of free radical activity as compared to probucol-untreated rats (Tables 7 and 12). A similar reduction in the formation of thiobarbituric acid reactive substances (TBARS) have been found by Parthasarathy and coworkers (1986) in Cu^{++} mediated oxidative challenge to incubated LDL's treated with probucol. One consequence of lipid peroxidation is a decrease in the fluidity of the lipid phase of the membrane assembly which may lead to deleterious effects on the major metabolic functions that are dependent on membrane structure and integrity (Slater et al, 1987). Further, as might be anticipated from its lipophilic properties, probucol is distributed in high concentration in cell membranes which mostly consist of phospholipid. Thus, it would be
strategically positioned to offer maximum protection to cell membranes and lipoproteins against in vivo peroxidative damage. It is possible that the generation of free radicals could lead to peroxidation of lipids and induction of biosynthesis of collagen which, in turn, could cause fibrosis and pathological conditions associated with ethanol-induced cirrhosis.

**Total Collagen Content**

This study found a significant increase in total collagen content in the liver as determined by hydroxyproline levels, in chronically ethanol-treated rats relative to the other treatment groups (Table 8). This tends to suggest that the ethanol-treated rats did show the initial stages of cirrhosis. Feinman and Lieber (1972) reported similar increases in collagen accumulation in the livers of chronically ethanol-treated rats and baboons. Chen and Leevy (1975), and Perier and coworkers (1984) observed that liver biopsy specimens taken from patients with alcoholic hepatitis and ethanol-induced cirrhosis exhibited a significant increase in collagen biosynthesis. The degree of protection offered by probucol against ethanol-induced collagen was found to be statistically significant (Table 8 and Figure 8). Since the fibrotic processes are the result of both the synthesis and degradation of collagen, then perhaps probucol prevents collagen increases in ethanol-treated rats by allowing collagen to maintain degradability. This is supported by the collagenase-proteinase K digestion data.
Collagenase-protease K soluble and insoluble collagen

The amount of collagenase-protease K enzyme soluble collagen was lower in ethanol-treated rats when compared to other treatment groups (Table 10 and Figure 10). This indicates that the amount of collagen available for \textit{in vivo} digestion by collagenase is greatly reduced. However, as observed in Table 8 and Figure 8, the total amount of collagen was found to be much higher in the ethanol-treated group compared to other treatment groups. This strongly suggests that most of the collagen present in the ethanol-treated group exists as enzyme insoluble collagen (Figure 11). The amount of insoluble collagen is significantly higher in ethanol-treated rats suggesting that ethanol or its metabolite may induce crosslinks which make it less enzyme digestible. The results of this study also suggest that probucol is able to prevent the formation of such crosslinks. One possible explanation of less soluble collagen leading to cirrhosis of the liver is that irreversible crosslinking may render the collagen to be enzymatically nondegradable and functionally inactive. This is possible because connective tissue structure is thought to be formed via self assembly, and any changes in normal chemical structure of the matrix components would lead to abnormal self assembly and perhaps fibrosis. Also, the lack of availability of soluble collagen may be interpreted as a collagen deficient state by the matrix cells and may lead to triggering of collagen biosynthesis.
Advanced Glycosylation Products

Advanced products of nonenzymatic glycosylation (AGP's) are slowly formed glucose derived compounds which are chemically irreversible and tend to accumulate continuously over time. AGP's have been considered to be responsible in the development of various complications associated with aging and diabetes.

This study found that AGPs were highest in the control group and the lowest in the probucol-treated groups (Table 9 and Figure 9). The 44% reduction of AGP's noted in livers of probucol-treated animals over controls strongly correlate with reduced levels of TBARS found in the same animals. This suggests that a major portion of the AGPs formed as a result of the protein crosslinking catalyzed by oxygen free radicals. Decreased AGPs in the ethanol treated rats may be due to acetaldehyde competitively interfering with sugar aldehydes, thus preventing AGP's. Normura and Lieber (1981) have reported covalent binding of exogenously added acetaldehyde to the proteins of liver microsomes and an even greater effect when acetaldehyde was generated in situ. Furthermore, this binding was significantly increased after long term ethanol consumption and paralleled induction of MEOS (microsomal enzyme oxidizing system). These results suggest a special capacity of native acetaldehyde to form covalent links with proteins associated with its production and may hint at possible involvement of free radicals in catalysis of the reaction. Acetaldehyde causes injury through the formation of protein adducts
which, in turn act as neo antigens (Israel et al, 1986) resulting in antibody formation, enzyme inactivation, decreased DNA repair, and alterations in microtubules, plasma membranes and mitochondria including swelling and abnormal cristae (Iseri et al, 1966, Lane and Leiber, 1966, Rubin and Leiber, 1967 a,b and Leiber and Rubin, 1968), with striking impairment of oxygen utilization. This may lead to even greater concentration of acetaldehyde and increased formation of acetaldehyde-collagen adducts and thus, leading to even more damage to the hepatic cells.

In conclusion, this study provides data which support the theory that oxidative stress is one of the important components in the development of ethanol-induced cirrhosis of liver. The data also suggest that some of the fibrotic signs noted in ethanol-induced liver damage may be reduced or even eliminated with the treatment of a potent antioxidant. The simultaneous reduction of both lipid peroxides and AGP's with the use of potent antioxidant indicates that AGP formation is largely an oxidative stress mechanism. Thus, this study tends to support the evidence that oxidative stress is involved in the development of a variety of diseased states, including ethanol induced cirrhosis of liver.
REFERENCES


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APPENDIX I
(Correlation matrices)
Table 12. Correlation matrix for the lipid peroxidation in the livers of various treatment groups$^a$.

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>C</th>
<th>A</th>
<th>PA</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>1.0</td>
<td>0.3</td>
<td>0.0001*</td>
<td>0.0001*</td>
</tr>
<tr>
<td>A</td>
<td>0.3</td>
<td>1.0</td>
<td>0.0001*</td>
<td>0.0001*</td>
</tr>
<tr>
<td>PA</td>
<td>0.0001*</td>
<td>0.0001*</td>
<td>1.0</td>
<td>0.187</td>
</tr>
<tr>
<td>P</td>
<td>0.0001*</td>
<td>0.0001*</td>
<td>0.187</td>
<td>1.0</td>
</tr>
</tbody>
</table>

C = Control; A = Ethanol; P = Probucol-treated; PA = Probucol-ethanol-treated.

$^a$ represents the probability that the data results between the treatment groups are the same.

* indicates the value of $p < 0.0001$.

Table 13. Correlation matrix for Total Hydroxyproline in the livers of various treatment groups$^a$.

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>C</th>
<th>A</th>
<th>PA</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>1.0</td>
<td>0.0001*</td>
<td>0.446</td>
<td>0.863</td>
</tr>
<tr>
<td>A</td>
<td>0.0001*</td>
<td>1.0</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>PA</td>
<td>0.446</td>
<td>0.001</td>
<td>1.0</td>
<td>0.128</td>
</tr>
<tr>
<td>P</td>
<td>0.863</td>
<td>0.001</td>
<td>0.128</td>
<td>1.0</td>
</tr>
</tbody>
</table>

C = Control; A = Ethanol; P = Probucol-treated; PA = Probucol-ethanol-treated.

$^a$ representing the probability that the data results between treatment groups are the same.

* indicates the value of $p < 0.0001$. 

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Table 14. Correlation matrix for Advanced glycated products (AGP’s) in the livers of various treatment groups\(^a\).

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>C</th>
<th>A</th>
<th>PA</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>1.0</td>
<td>0.004</td>
<td>0.001</td>
<td>0.002</td>
</tr>
<tr>
<td>A</td>
<td>0.004</td>
<td>1.0</td>
<td>0.001</td>
<td>0.022</td>
</tr>
<tr>
<td>PA</td>
<td>0.001</td>
<td>0.001</td>
<td>1.0</td>
<td>(0.0001^*)</td>
</tr>
<tr>
<td>P</td>
<td>0.002</td>
<td>0.022</td>
<td>0.001(^*)</td>
<td>1.0</td>
</tr>
</tbody>
</table>

C = Control; A = Ethanol; P = Probucol-treated; PA = Probucol-ethanol-treated. \(n = 6\) for all of the above treatment groups.

\(^a\) representing the probability that the data results between the treatment groups are the same.

\(^*\) denotes significance (\(p << 0.0001\)).

Table 15. Correlation matrix for collagenase-protease K enzyme soluble hydroxyproline levels in the livers of various treatment groups\(^a\).

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>C</th>
<th>A(^{*})</th>
<th>PA</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>1.0</td>
<td>0.0001(^*)</td>
<td>0.511</td>
<td>0.844</td>
</tr>
<tr>
<td>A</td>
<td>0.0001(^*)</td>
<td>1.0</td>
<td>0.003</td>
<td>0.011</td>
</tr>
<tr>
<td>PA</td>
<td>0.511</td>
<td>0.003</td>
<td>1.0</td>
<td>0.797</td>
</tr>
<tr>
<td>P</td>
<td>0.844</td>
<td>0.011</td>
<td>0.797</td>
<td>1.0</td>
</tr>
</tbody>
</table>

C = Control; A = Ethanol; P = Probucol-treated; PA = Probucol-ethanol-treated.

\(^a\) representing the probability that the data results between the treatment groups are the same.

\(^*\) indicates the value of \(p << 0.0001\).
Table 16. Correlation matrix for collagenase-protease K enzyme insoluble hydroxyproline levels in the livers of various treatment groups.

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>C</th>
<th>A</th>
<th>PA</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>1.0</td>
<td>0.001</td>
<td>0.362</td>
<td>0.916</td>
</tr>
<tr>
<td>A</td>
<td>0.001</td>
<td>1.0</td>
<td>0.0001*</td>
<td>0.0001*</td>
</tr>
<tr>
<td>PA</td>
<td>0.362</td>
<td>0.0001*</td>
<td>1.0</td>
<td>0.162</td>
</tr>
<tr>
<td>P</td>
<td>0.916</td>
<td>0.0001*</td>
<td>0.162</td>
<td>1.0</td>
</tr>
</tbody>
</table>

C = Control; A = Ethanol; P = Probucol-treated; PA = Probucol-ethanol-treated.

* representing the probability that the data results between the treatment groups are the same.

* indicates the value of p << 0.0001.

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APPENDIX II
(Standard curves)
Figure 12. Standard curve for determination of lipid peroxidation.
(TBA Assay)

Standard Curve (TBA Assay)

Absorbance

Conc. (mcg)

\[ r^2 = 0.992 \]
Figure 13. Standard curve for determination of total protein content. (Bradford Assay)

**Standard Curve (Bradford Assay)**

Absorbance (595 nm) vs. Total protein conc. (mcg)

$\Delta \ r^2 = 0.999$

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Figure 14. Standard curve for determination of hydroxyproline (HP) content.

Standard Curve (HP Assay)

Absorbance (557 nm)

Conc. (mcg)

\[ r^2 = 0.999 \]