Metabolic fate and toxic effects of one of the components of Tetradymia glabrata
by Sandra Keller Holian

A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Chemistry
Montana State University

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Date May 16, 1975

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SANDRA KELLER HOLIAN

A thesis submitted in partial fulfillment
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INTRODUCTION

Tetradymol is a toxic constituent isolated from Tetradymia glabrata by Dr. Sam Reeder. It is a member of the Compositae family, Senecio tribe, resembling sage brush and found in a broad region covering an area north to Washington, east into Wyoming, west to California and south to the Utah-Arizona border. It was first shown to be responsible for death in sheep on the Nevada ranges by Fleming.[1] Further, it was known at that time to cause a reversible phenomenon called "Big Head", the symptoms of which were facial and ear tissue swelling. For more information on "Big Head" the reader is referred to Brown.[2]

From 1918 to 1922 Fleming and his colleagues conducted feeding experiments and a brief chemical study in which the more pertinent facts necessary for killing sheep were ascertained: (1) under scarce food conditions sheep would eat the new growth of T. glabrata, normally they would not, and an adult sheep could eat up to 2% of its body weight per day without apparent harm; (2) since the lethal dose could be fed over a relatively long period it was thought the toxic principle was slowly eliminated; (3) death was attributed to hepatodysfunction and cardiac failure; (4) the toxic constituent was contained in petroleum ether and acetone extracts from the green plant.[1]

Considering this work and a later investigation by

Clawson and Huffman,[3,4] the problem of isolating the toxic constituent of T. glabrata was undertaken by Drs. S. K. Reeder[5] and J. C. Hurley.[6] Two toxic compounds were isolated, tetradymol and tetradymadiol 6-isobutyrate, the following is a brief summary of Reeder's[5] work with tetradymol.

From whole plant feeding experiments on sheep it was shown that: (1) feeding 1% of body weight for three days resulted in death; (2) bromsulphalein clearance time was greatly lengthened; (3) blood serum ammonia levels were elevated three to six times in poisoned sheep.[5] The last two points indicate hepatodysfunction. To test cardiac dysfunction electrocardiograms were monitored on all sheep resulting in no marked changes being observed.

Autopsies were performed on all sheep that were poisoned. The results are summarized below:[6]

1. Liver tissue demonstrated panlobular necrosis localized in the centralobular area.
2. Kidney tissue showed some general congestion and swelling and hyperemia especially in the medulary portion.
3. Varying degrees of congestion were reflected in the lungs with some emphysema and bronchiolar hemorrhage.
4. Cardiac tissue was not greatly different from

normal revealing some congestion and a few subepicardial hemorrhages.

It was concluded that the toxic principle was a hepatotoxin and did not greatly effect the heart.

The results of plant-extract feedings are shown in Table 1.[7] This Table shows the percentage of plant weight to body weight was similar to whole-plant feedings and for the hexane or acetone extracts, the BSP clearance time and blood serum ammonia level changes were comparable. Results of feeding hexane or actone extracts were similar in dosage level and hepatic damage incurred indicating the toxic constituent was successfully extracted by these solvents.

Since sheep were a large and expensive laboratory animal, other smaller animals were tested resulting in similar gross changes in the livers. It was decided that mice would be used for further toxicity experiments.

Preliminary separation and feeding experiments of the crude extract with mice indicated two different toxins. One of the toxins, tetradymol, was isolated and its structure was confirmed by X-ray crystallographic determination of the mercuric chloride derivative shown in Figure 1.[8]

Extractions with hexane were made with both ground and unground plant material. Since grinding the material did not result in the isolation of more tetradymol, it was assumed the

TABLE 17

Plant Extract Sheep Feeding Experiments

Sheep number	Wt. lbs.	Age yrs.	Extract fed	Extract from pounds	% plant of body weight	NH_4^+ level*	BSP clearance°	Result
H-636	98	1	Acetone	4.4	4.5	3/12 5/18	3/36 5/79	death
H-665	104	1	Hexane	5.0	4.8	3/14 5/13 7/14	5/44 7/39	v. ill°° sacrificed
H-634	103	1	Pentane of ethanol	2.5	2.5	3/6		release
H-628	104	1	Ethanol remains	2.5	2.5	3/6		release
			Plant extracted with	pounds fed				
H-641	102	1	Acetone	2.2	2.2	3/5		re-use
H-671	90	1	Hexane	2.5	2.8	3/8		release
H-653	100	1	Ethanol	2.5	2.5	3/6		release

*Recorded as a fraction with the day of the feeding experiment when the NH_4^+ level test was run in the numerator and the g/ml of NH_4^+ found in the serum recorded as the denominator

°Recorded as above with the day in the numerator and the $T_{\frac{1}{2}}$ in minutes recorded as the denominator.

°°V. ill meaning very sick, actually down and on the verge of death.

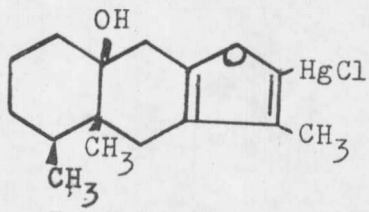


Figure 1. Tetradymol mercuric chloride[8]

toxin was a surface compound and further hexane extractions were made on unground plant material.

An LD_{50} of 170 to 333mg/kg was determined for the pure tetradymol compound by feeding experiments with mice. The results are shown in Table 2.[9]

Because preliminary feeding experiments indicated tetradymol was a hepatotoxin, the liver would be an important organ in considering what happens to tetradymol and the effects of tetradymol poisoning. Therefore, a brief review of the liver structure and function will be presented.

The liver contains hundreds of lobules which are the basic functional unit. These lobules are basically hexagonal in shape being longer than wide. The liver lobule is constructed around a central vein and is composed principally of many hepatic cellular plates. The plates are usually two cells thick and radiate centrifugically from the central vein. Lying between the hepatic plates are the small bile canaliculi and around the plates are the liver sinusoids. On the periphery of the lobule are portal areas which contain the bile

TABLE 2[9]

Mouse Feeding Experiments

Materials and/or carrier	mg/kg of toxin	ml/kg total volume	Number of animals	% dying in one week
1. Pure (II)* 50% ethanol in n-hexane	460	3.8	9	25
2. "	360	3.6	8	50
3. "	280	3.5	8	63
4. "	190	3.1	8	75
5. "	160	4.0	8	50
6. 50% ethanol in n-hexane	0.0	3.3	8	50
7. "	0.0	5.0	9	50
8. Crude extract in propylene glycol	2000-3000	3.0-4.5	5	00
9. Crude extract in n-hexane	2100-3300	4.4-5.8	12	100
10. "	1100-1200	1.9-2.0	4	25
11. N-hexane	0.0	4.0-4.9	8	00
12. "	0.0	7.0-8.6	7	00
13. Sublimed (II) in n-hexane	750-580	4.8-6.2	7	100
14. "	330	3.3	9	100
15. Pure (II) in n-hexane	330	3.3	9	100
16. "	170	3.3	11	45
17. "	282	3.9	5	40
18. "	200	3.9	5	00
19. "	140	3.9	5	00
20. "	100	3.9	5	00

*Pure (II) refers to sublimed, base washed tetradymol.

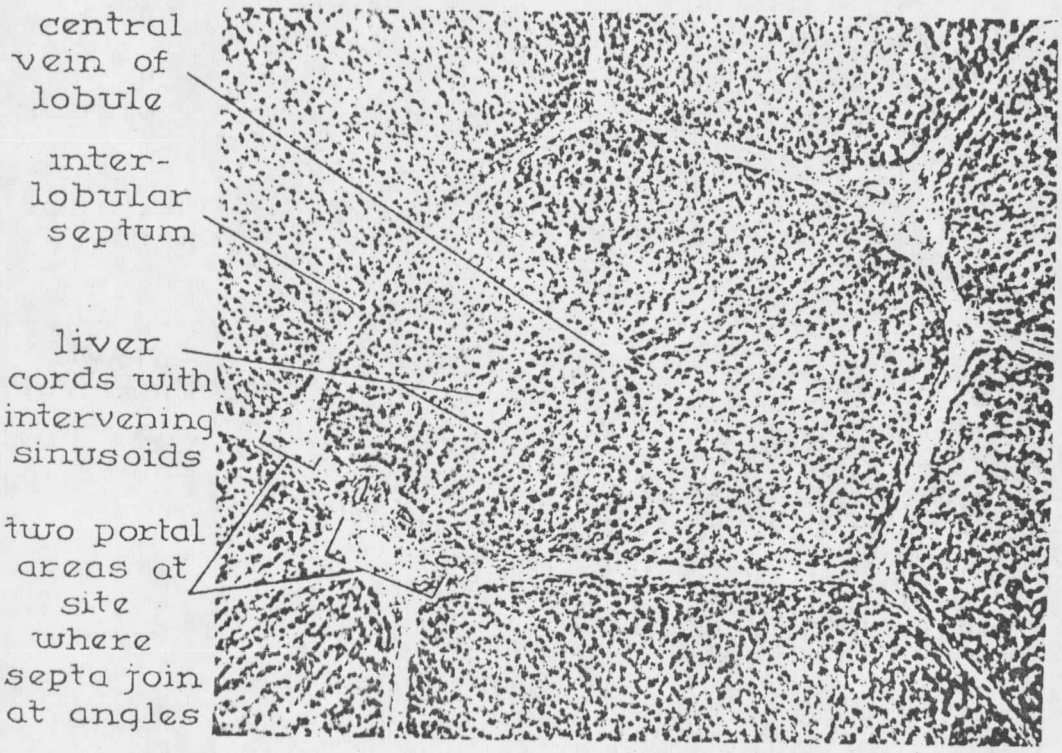


Figure 2. Low-power photomicrograph illustrating the classic hexagonal lobule.[10]

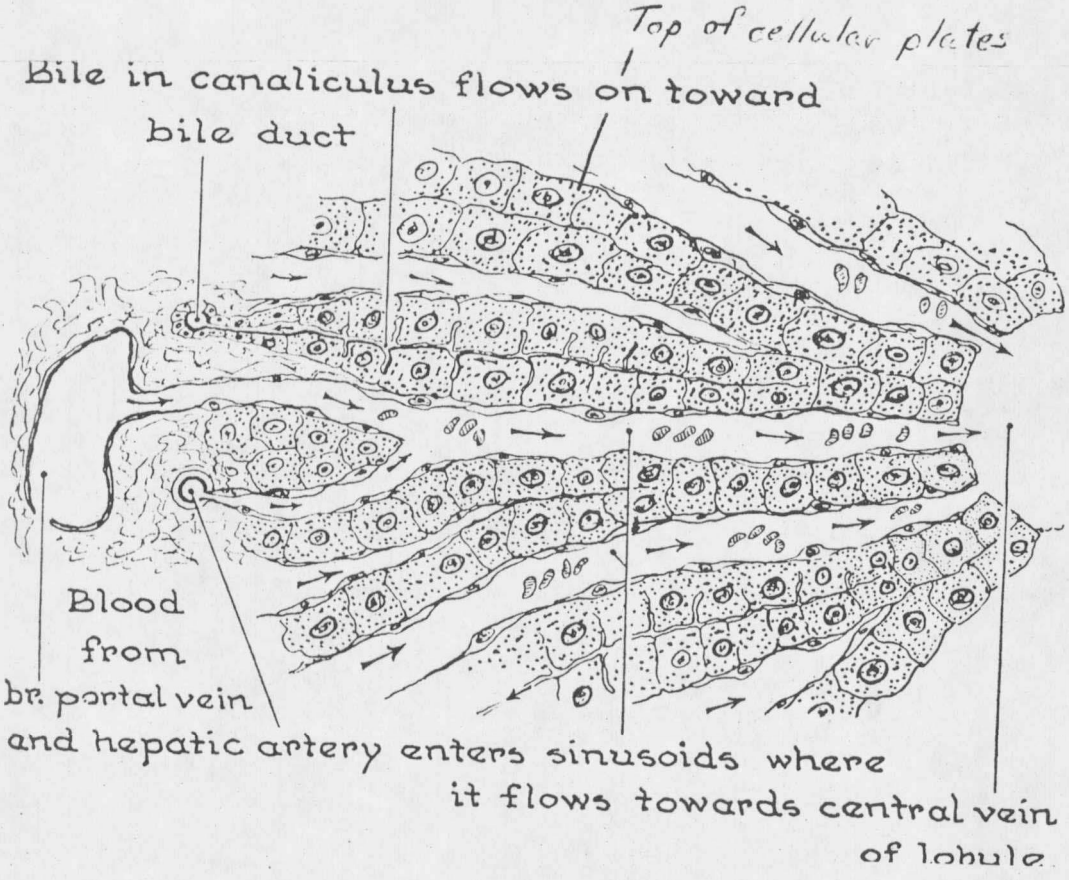


Figure 3. Blood and exocrine flow in liver lobule.[12]

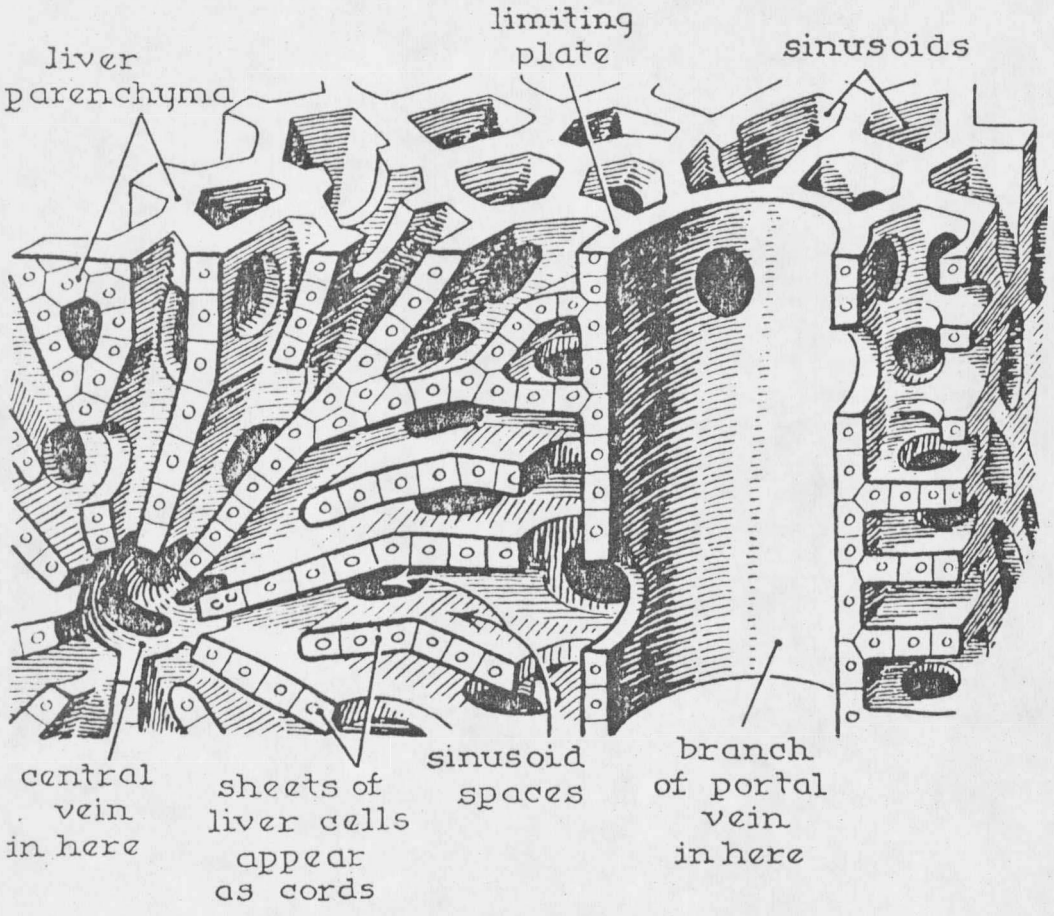


Figure 4. Quadrant of a liver lobule.[13]

duct, hepatic artery, and portal vein.[10,11] Three views of the liver lobule are illustrated in Figures 2, 3, and 4.[10,12,13]

The liver lobule has a dual blood supply: (1) blood flows in from the portal vein through the liver sinusoids, empties into the hepatic veins and hence into the vena cava (this food-laden blood comes from the intestine); (2) oxygen rich blood flows in from the hepatic artery through the sinusoids and empties into the hepatic veins.[11,14,15]

The exocrine secretions of the hepatic cells are drained away by the bile canaliculi which carry the secretions from the central area of the lobule to the bile duct and is eventually emptied into the intestine.[16,17]

The functions of the liver are numerous and intricate; therefore, only a brief summary will be presented.

The hepatocytes can store a variety of compounds; such as, glycogen, amino acids, proteins, iron and vitamins. These can then be released into the system when they are needed.[18,19] As a result the liver then performe not only a storage function, but also, a regulatory function. For example, the liver takes glucose from the blood and stores this as glycogen. When glucose concentration begins to fall it is returned to the blood, which is called the glucose buffer function of the liver.[19,20,21] Another way the liver maintains normal blood glucose levels is through gluconeogenesis which converts amino

acids and lactic acid to glucose.[20,21]

The liver performs important functions in protein metabolism; (1) deamination of amino acids; (2) formation of urea for ammonia removal; (3) formation of plasma proteins; (4) interconversions; such as, transaminations, among the different amino acids and other compounds.[18,19]

The liver has a protective function in detoxifying various compounds. One example is the transformation of ammonia to urea. Further, the liver will transform and/or conjugate undesirable products or compounds that are absorbed from the intestine which otherwise might prove deleterious to the body.[19,20]

A wide range of compounds comprise the collection of hepatotoxic agents ranging from simple molecules, such as, carbon tetrachloride to such complex compounds as steriods. Their deversity is reflected not only in their structure but also in their modes and degrees of action. The following discussion is to aid in identifying and classifying these agents into a more unified system.

In Table 3, hepatotoxic agents are classified by the presumed mechanism of hepatotoxicity.

The criteria for distinguishing between the types of hepatotoxins listed above are summarized in Tables 4, 5, and 6. The compounds that are intrinsic hepatotoxins were

Table 3[20]

Mechanistic Classification of Hepatotoxins

I. Intrinsic hepatotoxins

   A. Direct - injure liver cells directly and other organs

   B. Indirect - injure liver cells by diverting, blocking, or competitively inhibiting essential metabolites

II. Host Idiosyncracy

   A. Hypersensitivity

   B. Metabolic abnormality in host

Table 4[22,23]

Direct Hepatotoxicity Criteria

1. Brief interval between exposure and liver damage
2. Toxicity dose related
3. Distinct liver lesions and often other organs
4. Experimentally reproducible
5. High incidence
6. Protoplasmic poisons
7. Histological change is the same in man and predictable from animal experiments

Table 5[24]

Indirect Hepatotoxicity Criteria

1. Same as for Direct except for 3 and 6
2. Hepatic necrosis or other damage produced by:
   a. Competition with essential metabolites
   b. Selective binding of essential metabolites or nutrients
   c. Inhibition of specific enzyme functions
3. Selective interference with hepatic secretory or excretory mechanisms without parenchymal damage

Table 6[25,26]

Hypersensitivity Criteria

1. Sensitization period (1-4 weeks) or previous exposure
2. Recurrence of liver damage on readministration
3. Cross-, hypo-, or desensitization may be produced
4. Dose independent
5. Low incidence of occurrence
6. High incidence of rash, fever, eosinophilia
7. Coincidence of blood dyscrasins
8. Histology consistent with hypersensitivity
9. Injury not produced in animal species studies

originally grouped in a large class of industrial chemicals or solvents, these being such things as carbon tetrachloride, chloroform, halogenated hydrocarbons, etc.[27] In addition, some drugs have been shown to cause direct hepatotoxicity.

A brief summary of necrosis will be presented before discussing compounds that cause liver damage.

Hepatic necroses may be roughly classified by distribution: (1) focal necrosis, ie., small necrotic foci distributed without any constant relationship to particular areas of the liver lobules; (2) zonal necrosis, in which the involved areas are in fairly constant relationship to a particular part of the liver lobules and are referred to as central, midzonal, and peripheral; (3) diffuse necrosis in which hepatic parenchyma cells are destroyed over massive areas.[28]

In central or centralobular necrosis, the most common of zonal necroses, the lobule surrounding the central vein is necrosed. This form of necrosis may be caused by a variety of chemical poisons, such as, carbon tetrachloride, chloroform, and trinitrotoluene.[29] Midzonal necrosis involves the middle regions of the liver lobule and is apparent with yellow fever.[29] Phosphorus poisoning causes peripheral necrosis which is localized around the portal area of the lobule.[29]

The first sign of necrosis is swelling in the cells where more severe changes will be apparent later. This will be

followed by cytoplasmic vacoular degeneration or fatty infiltration. Nuclear changes will become apparent such as karyolysis, pyknosis, and karyorrhexis and cell definition will be lost as the cell dies.[30]

Resistance of hepatic cells varies with their metabolic and nutritional state. Adequate stores of glycogen or adequate amounts of methionine, choline or other vitamin B_{12} complex components appear to give some protection from injurious influences.[28]

As stated earlier numerous chemicals cause liver injury but little is known of the mechanism by which these chemicals produce such injury.[31] The next section is a discussion of some compounds that produce liver necrosis (classification, distribution), how necrosis may be altered and a suggestion of why necrosis occurs.

In 1961, Denz and Hanger[32] isolated and characterized the liver toxin from the leaves of the Ngaio tree (Myoporum Letum). Various domestic animals would readily eat the tree's leaves, the result of which was fatal. This poisoning resulted in liver damage, icterus, and photosensitivity. The toxic principal was identified as a sesquiterpene ketone called ngaione; the structure is given in Figure 5.[32] This compound was isolated from the Ngaio oil. The LD_{50} of the oil was 510mg/kg in mice. The toxin caused zonal liver necrosis,

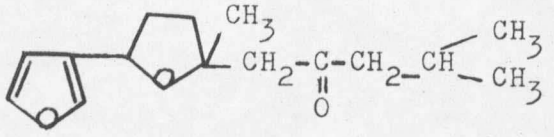


Figure 5. Structure of Ngaione[32]

usually midzonal, and death in mice. The compound's LD_{50} was demonstrated to be 300mg/kg, a hepatotoxin of moderate toxicity.

Seawright and O'Donahoo,[33] in 1971, did a more complete study of the histology of ngaione. They administered intragastrically a LD_{50} dose of ngaione then sacrificed four mice at each time period, 1, 3, 6, 12, 24, and 48 hours after poisoning. Under the light microscope midzonal damage was not seen until three hours, with the electron microscope midzonal changes were present in one hour. The damage visible by light microscope started as fine cytoplasmic vacoules and progressed through to complete necrosis by twelve hours. After this some regenerative changes were apparent as indicated by phagocytic activity of macrophages and the livers were apparently normal after nine days.

In 1972, Seawright and Hrdlicka[34] reported that pretreating with phenobarbital, an inducer of mixed function oxidase enzyme synthesis, or SKF-525A, a binding inhibitor of

cytochrome P-450, effectively changed the LD_{50} of ngaione and changed the zonal necrosis in mice. After pretreating with phenobarbital the LD_{50} was increased to 370mg/kg and the necrosis was moved from midzonal to peripheral. Pretreating with SKF-525A increased the LD_{50} to 530mg/kg and changed the necrosis to the centralobular region. Seawright concluded from these results there was a concentration gradient of the mixed function oxidase enzymes in the lobule and a critical concentration was necessary for the toxic effects. In view of this, SKF-525A moved the critical concentration closer to the central area, by binding to the peripheral and midzonal cytochrome P-450, resulting in the necrosis being reflected in the centralobular area. Phenobarbital, by inducing enzyme synthesis, moved this critical concentration to the peripheral region and resulted in necrosis in this region. For hepatotoxic damage to occur a critical concentration ratio of toxin to mixed function oxidase enzymes (cytochrome P-450) was necessary.

After reports that overdoses of acetaminophen caused hepatic necrosis in man, Mitchell, et al.,[35] examined the histology and the results of various pretreatments on this necrosis. The structure of acetaminophen (4-hydroxyacetanilide) is given in Figure 6.

Acetaminophen caused centralobular necrosis in mice and

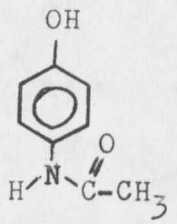


Figure 6. Structure of acetaminophen

rats similar to that in man and was shown to be dose dependent. By pretreating with phenobarbital the incidence and severity of necrosis was potentiated. Piperonyl butoxide, a binding inhibitor of cytochrome P-450, and cobaltous chloride, an inhibitor of mixed function oxidase enzyme synthesis, when used in pretreating the animals prevented necrosis. The effect of pretreatment on the rate of acetaminophen disappearance from liver and plasma showed no alteration with phenobarbital and cobaltous chloride but was slowed with piperonyl butoxide. Mitchel concluded a metabolite covalently bound in the liver macromolecules caused the liver necrosis and that pretreating resulting in alteration of the necrosis also altered binding of the metabolite.[35,36]

Mitchell, et al.,[37] examined the possibliity of glutathione protection of acetaminophen necrosis by pretreating with cysteine, an inducer of glutathione synthesis, or diethylmaleate, a binding inhibitor of glutathione. Pretreatment with cysteine reduced necrotic damage and diethylmaleate pretreatment potentiated the necrosis indicating a

protective role by glutathione. Mitchell concluded that the metabolite did not cause necrosis until glutathione availability was depleted by conjugation of the metabolite.

Brodie[38] suggested in 1967 that chemically inert chemicals may cause necrosis by a covalent linkage between a metabolite and various macromolecules. Brodie, et al., supported his suggestion, in 1970, by showing that ^{14}C-bromobenzene was covalently bound at sites of necrosis.[39] They concluded that the liver can convert stable organic compounds to toxic agents which cause necrosis by covalent bonding. They showed the importance of microsomal enzymes in this conversion by pretreating with phenobarbital, potentiating necrosis, and SKF-525A, decreasing necrosis caused by bromobenzene. Later in 1971, Mitchell, et al.,[38] published a report supporting Brodie's conclusions.

In 1973, Zampaglione, et al.,[40] continued to show the role of detoxifying enzymes in bromobenzene necrosis. Pretreating with phenobarbital increased bromobenzene metabolism and potentiated necrosis, conversely, SKF-525A pretreatment slowed metabolism and prevented necrosis. The interesting point of this publication was the effect of 3-methylcholanthrene pretreatment, an inducer of mixed function oxidase enzyme synthesis. Although 3-methylcholanthrene increased the metabolism of bromobenzene, it provided protection against

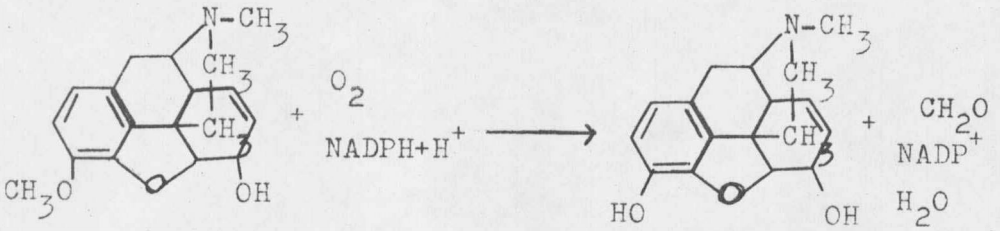
the necrosis. According to Zampaglione, et al., this protection resulted from an alteration in the pathways of bromobenzene metabolism.

During the previous discussion it is apparent that the mixed function oxidase enzyme system, specifically cytochrome P-450, is intimately involved in the toxicity of chemicals. The following is a review of cytochrome P-450 as to mechanism, multiplicity, effectors, and the spectroscopic properties.

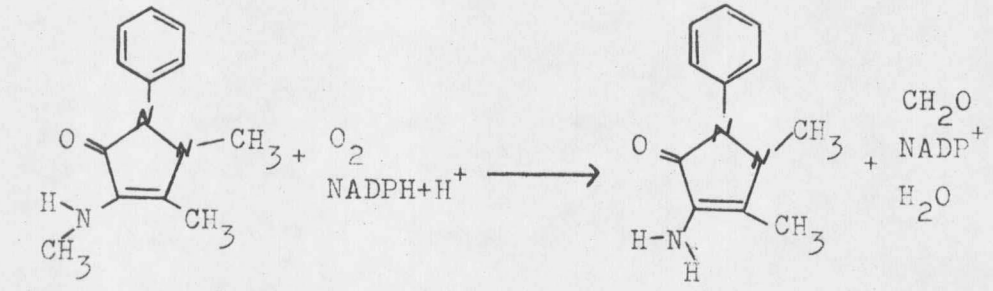
When foreign compounds, particularly lipophilic compounds, are introduced to the body and go into the liver they may be oxidized by the mixed function oxidase system. Cytochrome P-450 is considered to be a terminal hydroxylase in this system. Cytochrome P-450 can catalyze a number of mixed function oxidase reactions, three of which are illustrated in Figure 7.

The diversity of chemicals modified by cytochrome P-450 catalyzed oxidations is staggering and include conversions such as: (1) multiple positions of hydroxylation on steroid molecules; (2) oxidative conversion of heme to bile pigments; and (3) omega oxidation of fatty acids.[41] In table 7 are representative substrates for cytochrome P-450 catalyzed reactions and their reaction pathways.

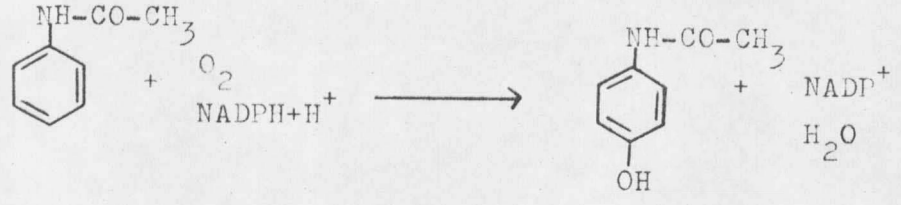
The function of cytochrome P-450 is to activate molecular oxygen for introduction into a compound resulting in a more



Codeine Morphine



Monomethyl-4-aminoantipyrine 4-Aminoantipyrine



Acetanilide p-Hydroxyacetanilide

Figure 7. Three types of mixed function oxidation reactions.41

Substrates and Pathways for Cytochrome P-450[42]

Pathway	Substrate
Aromatic Hydroxylation	3,4-benzpyrene Zoxazolamine Acetanilide Estradiol
Aliphatic Hydroxylation	Hexobarbital Testosterone Fatty Acids
N-Dealkylation	Aminopyrine Meperidine Imipramine
O-Dealkylation	Codeine Acetophenitidin
S-Dealkylation	Chlorpromazine

polar product. Intensive efforts have been made to elucidate the mechanism by which oxidative transformations catalyzed by cytochrome P-450 occur. Estabrook has proposed the following six steps as occurring in the reduction and oxidation of cytochrome P-450.[43]

1. The reversible interaction of a substrate molecule with a low spin form of ferric cytochrome P-450 accompanied by the formation of a high spin form of the ferric-substrate complex of cytochrome P-450;
2. The one electron reduction of the high spin form of the ferric-substrate complex of cytochrome P-450 to a ferrous-substrate complex.
3. The reversible interaction of oxygen with the ferrous-substrate complex of cytochrome P-450 to form an oxygenated or oxy-ferrous-substrate complex.
4. A second one electron reduction step required to

generate an intermediate which is as yet undefined;

5. A proposed rearrangement accompanying internal oxidation and reduction reactions resulting in the introduction of one atom of molecular oxygen into the organic substrate in the form of a hydroxyl group concomitant with the release of the other atom of oxygen as water;

6. The dissociation of the hydroxylated product from ferric cytochrome P-450 with the regeneration of a low spin form of ferric cytochrome P-450.

It has been demonstrated by several researchers that an iron-sulfur protein plays a role in the two electron transfer steps required for bacteria and adrenal cortex cytochrome P-450 function. This has not been well documented with microsomal bound cytochrome P-450. A proposed pathway for electron transfer and resulting substrate oxidation is shown in Figure 8.

Early evidence suggested there was more than one form of cytochrome P-450. This indication was obtained by visible spectroscopy and electron paramagnetic resonance spectroscopy by observing changes involved in the binding of different ligands to cytochrome P-450. This indicated two forms that were spectrally and enzymatically distinct, cytochrome P-450 and P448 ($P\text{-}450_1$). Further evidence was obtained by combined potentiometric and electron paramagnetic resonance titrations of cytochrome P-450 from phenobarbital-treated rats indicating three forms of cytochrome P-450. One was a high spin

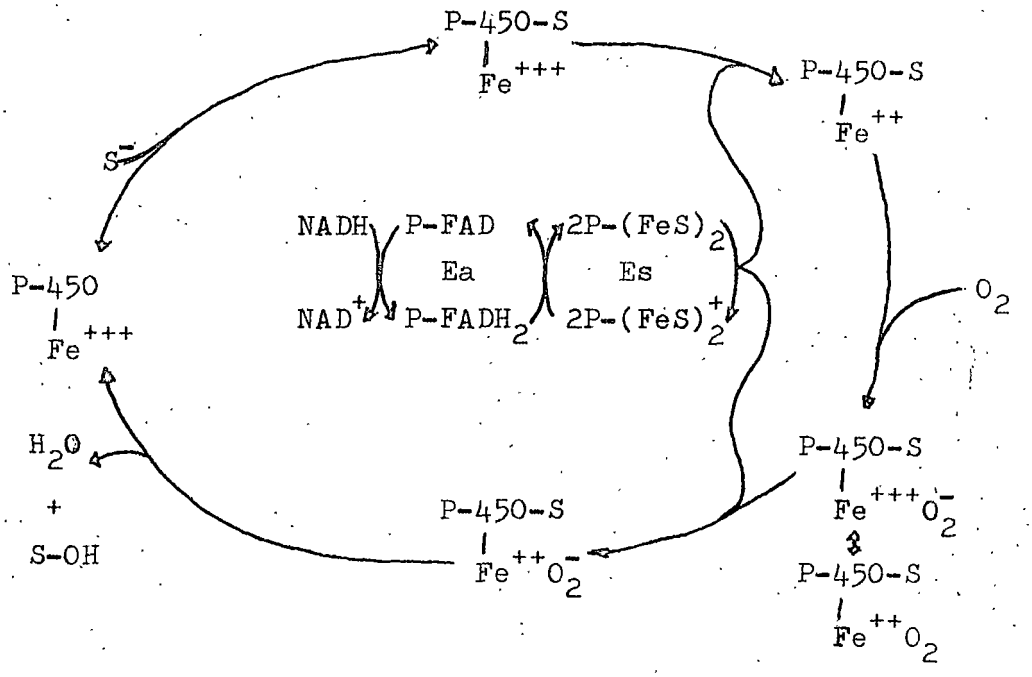


Figure 8. Proposed pathway for electron transfer and substrate oxidation.[44]

hemoprotein, referring to the iron, and the other two were low spin hemoproteins. Comai and Gaylor[45] identified three forms of cytochrome P-450 qualitatively and quantitatively by visible spectral changes that occurred when combining with various ligands. The forms were separated on a Whatman DE-52 ion exchange column or on a Sigmadiethylaminoethylcellulose column. The forms had different binding affinities for the different ligands. The ligands used were cyanide, carbon monoxide and octylamine.

Various pretreatments altered the relative amounts of the three forms. 3-methylcholanthrene pretreatment increased

Form III, Form II was increased by phenobarbital pretreatment and pretreatment with ethyl alcohol preferentially induced Form I.

Walton and Aust[46] later resolved three forms of cytochrome P-450 from rat liver microsomes by SDS-polyacrylamide gel electrophoresis. They obtained three bands representing hemoproteins having molecular weights of 53,000, 50,000, and 44,000. Pretreatment with 3-methylcholanthrene increased the level of the 53,000 molecular weight species (Form III), phenobarbital pretreatment induced the level of the 44,000 molecular weight species (Form II), and the 50,000 molecular weight species (Form I) was the major hemoprotein in the controls.

As suggested above a variety of chemicals affect the characteristics of the mixed function oxidase enzyme system. Some of these compounds are: (1) phenobarbital[47,48,49,50] alters mixed function oxidase activity; (2) 3-methylcholanthrene[47,48,49,50,51] alters mixed function oxidase activity; (3) substrates,[52,53] such as, aminopyrine, ethylmorphine, and other polycyclic hydrocarbons, alter the metabolic rate of the mixed function oxidase system; (4) SKF-525A and its congeners[54] inhibit mixed function oxidase activity by binding to cytochrome P-450; (5) methylenedioxybenzenes[55] inhibit the activity of the mixed function oxidase system by

binding to cytochrome P-450; (6) cobaltous chloride[56] administration inhibits the synthesis of mixed function oxidases; and (7) 1-arylimidaxoles[57] have recently been shown to be inhibitors of mixed function oxidase activity.

From the discussion of cytochrome P-450 multiplicity and the discussion of effectors of mixed function activity, it is apparent that phenobarbital and 3-methylcholanthrene not only induce different spectral[47,51] and catalytic[48,49,50] forms of cytochrome P-450 but also induce different cytochrome P-450 hemoproteins.

Chemicals are not the only modifiers of mixed function oxidase activity. It has been shown that improper storage[58] can reduce the "in vitro" activity of this enzyme system. Litterest, et al.,[58] isolated microsomal pellets and froze them for various periods of time, 24 hours up to 20 days. There was no significant lose of activity up to 10 days. Preparation of the pellet for freezing can alter the stability and activity of this system.[59] Burke and Bridges[59] reported that the best storage method was a microsomal pellet overlaid with buffer. If the microsomes were resuspended and then frozen some stability was lost.

Varying the "in vitro" assay conditions for cytochrome P-450 can affect the stability and quantitation measurements.[60] The best measurement of cytochrome P-450 activity was

achieved when the protein content was held at approximately 2.0 to 2.5mg/ml in 0.1M phosphate or tris buffer at a pH range of 6.6 to 7.0 and without KCl being present.

In 1964, Omura and Sato[61] established a procedure for measuring the cytochrome P-450 content of liver microsomes spectroscopically. This method is commonly used today. In this procedure, microsomes are isolated and suspended in a 0.1M phosphate buffer at a protein concentration of 2mg/ml. The microsomal preparation is placed in a reference and a sample cuvette. The microsomes in the sample cuvette are reduced by adding sodium dithionite and CO is bubbled through the sample for approximately 20 seconds. The difference spectra are run on the microsomal preparation resulting in a reproducible peak at 450nm. The cytochrome P-450 concentration is obtained by taking the difference in absorbance between 490nm and 450nm and using the molar extinction coefficient of $91 cm^{-1} mM^{-1}$.[62]

The spectral properties of reduced microsomes may be influenced by pretreating the animals with various compounds, such as, phenobarbital[51,63,64] or 3-methylcholanthrene.[51,63,] This pretreatment can change the location of the peak, ie. 450nm to 448nm, and usually results in an increase in the concentration of cytochrome P-450.

Different spectral properties of cytochrome P-450 are

reflected in the binding characteristics of non-reduced microsomes with a variety of compounds. In 1966, Remmer, et al.,[65] reported that when various substrates were added to liver microsomes two types of spectral changes resulted. One spectral change, termed Type I, was characterized by a trough at 420nm and a peak at 385nm. The other spectral change, termed Type II, has a peak at 430nm and a trough at 390nm. In Table 8[66,67,68] is a list of compounds that cause such spectral changes.

Table 8[65,66,67]

Compounds Causing Type I or II Spectral Changes

Type I	Type II
Hexobarbital	Aniline
Phenobarbital	DPEA
SKF-525A	Nicotine
Piperonyl butoxide	Nicotinamide
Aminopyrine	Pyridine
Amobarbital	p-aminophenol
DDT[d]	Cortisol*
Chlorpromazine	Coricosterone*
N,N-dimethylaniline	Acetanilide*
Testosterone	Ethylisocyanide*

* These compounds give a modified Type II spectral change, characterized by a shifting of the 430nm peak.

Narasimhulu[68] studied spectra changes in relation to the steroid C-21 hydroxylation system. He found that the Type I spectral change was rapid and proceeded hydroxylation. He concluded that a Type I spectral change reflected the amount of cytochrome P-450 activated for redox reactions.

Soliman, et al.,[69] investigated the inter-relation of the Type I and Type II binding sites. By observing various displacements caused by one Type on the other, he concluded the sites were inter-related. Drug binding affected both sites and the extent of mutual displacement was not dependent on spectrum produced but on the dissociation constants of the drugs.

Another way the liver handles foreign compounds, principally the lipid soluble compounds, is to conjugate them with glutathione or UDP-glucuronic acid. This gives a more water soluble product and can take place before or after metabolism through the mixed function oxidase system. The conjugated product is usually considered to be non- or less toxic than the nonconjugated form.[70,71,72] The conjugation shemes for the two conjugators are presented in Figures 9 and 10.

Pretreating with cysteine or diethylmaleate changes the effective concentration of glutathione available for conjugation. Cysteine is an enhancer of the synthesis of

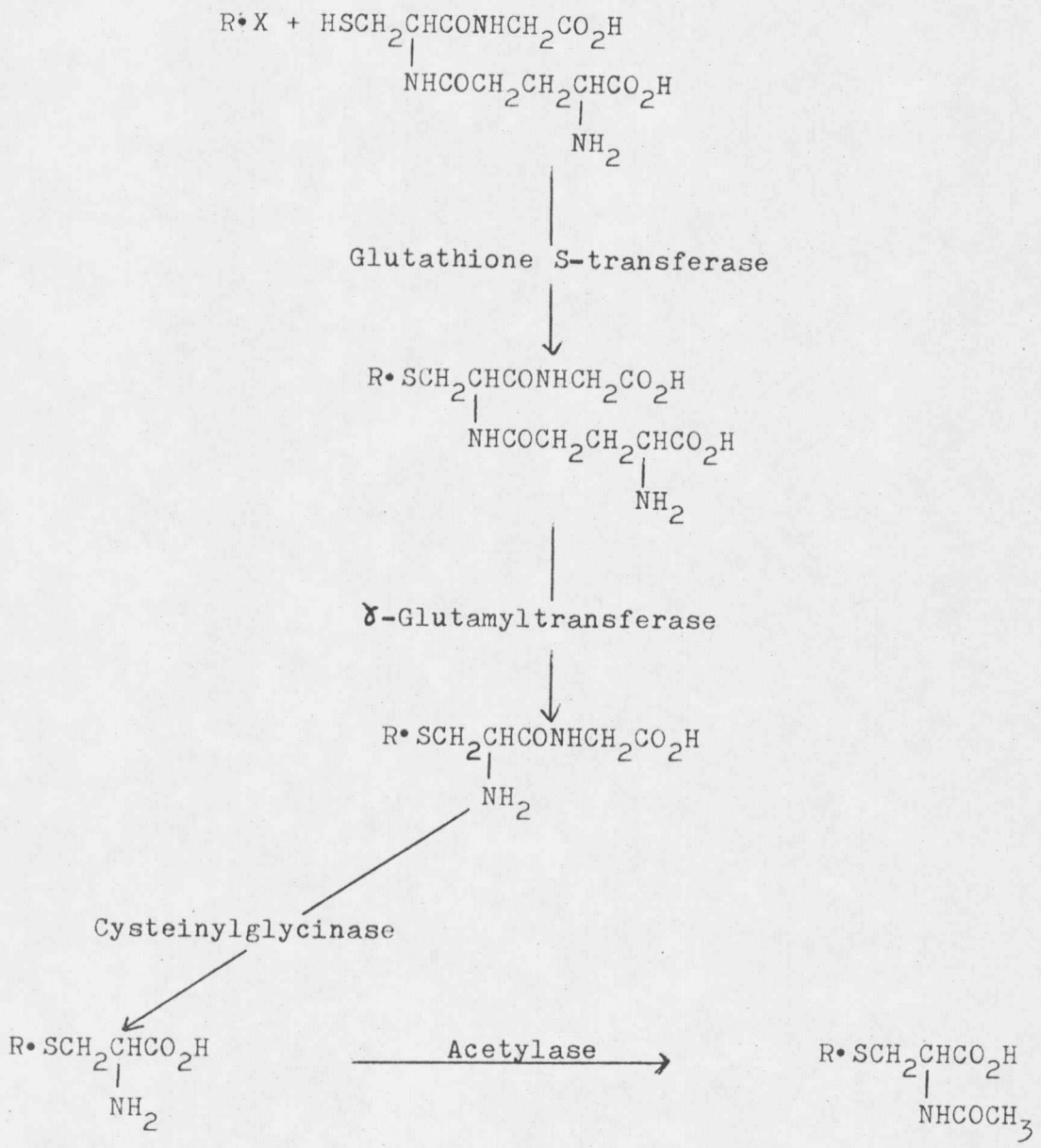


Figure 9. Conjugation scheme for glutathione.[70]

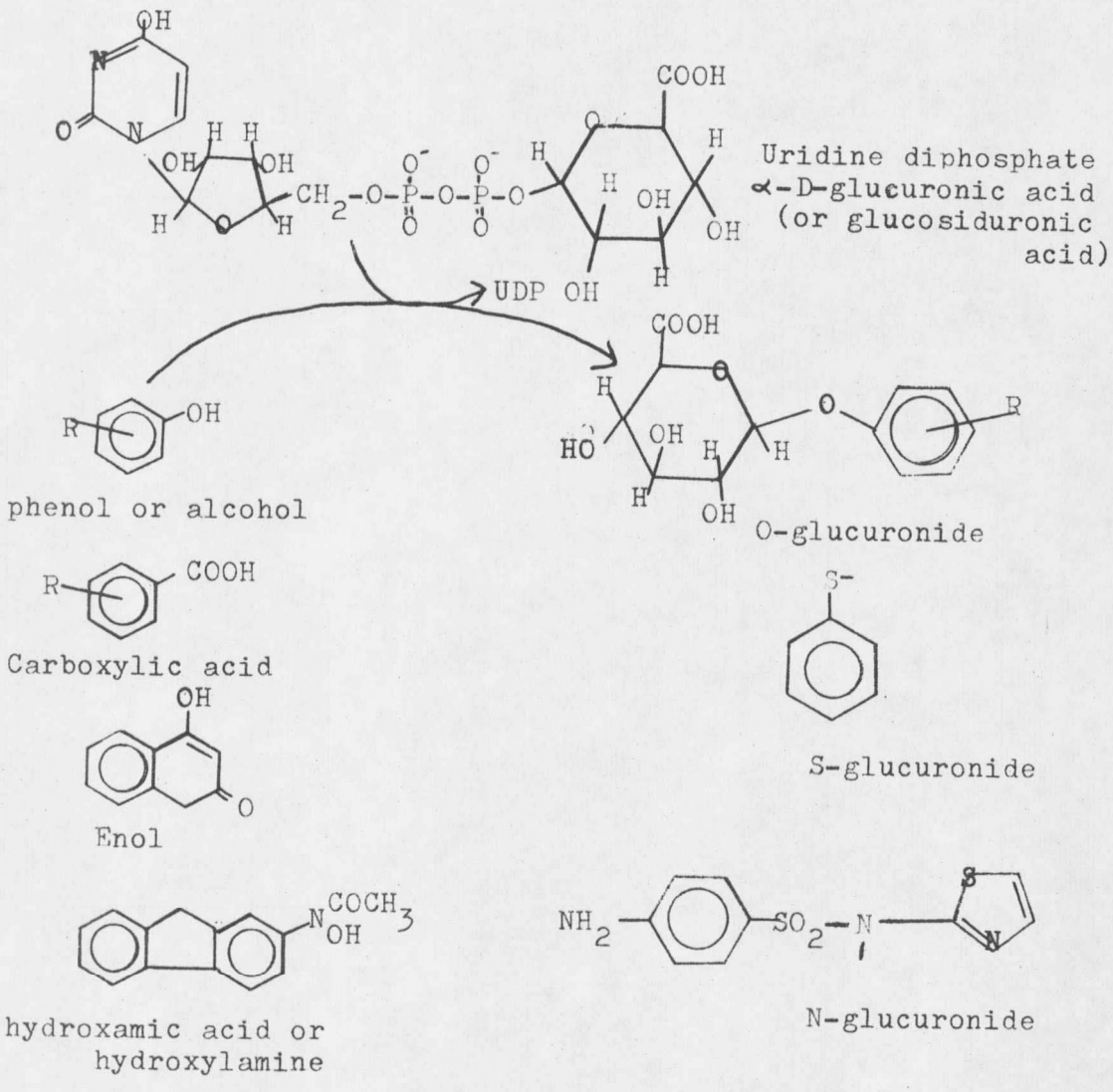


Figure 10. Conjugation scheme for UDP-glucosiduronic acid.[76]

glutathione.[73] It is the middle amino acid of glutathione, Figure 11. Diethylmaleate is a binding inhibitor of glutathione.[74]

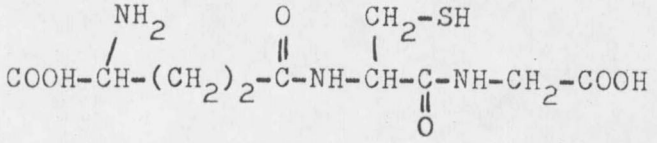


Figure 11. Structure of glutathione

Salicylamide is a binding inhibitor of UDP-glucuronic acid[75] and would reduce the amount of conjugation with another compound.

Taking into consideration the previous information on how the liver handles foreign compounds, what effects can be seen from hepatotoxins, and how these effects can be altered by various pretreatments study was conducted on the hepatotoxin, tetradymol.

RESULTS AND DISCUSSION

Before "in vivo" or "in vitro" research with tetradymol could begin an accurate method of quantitation the toxin had to be devised. The usual approach to this problem is to radiolabel the compound with either tritium or ^{14}C-carbon. After labeling tetradymol with tritium the resulting ^{3}H-tetradymol proved to be too intractable to isolate.

Ehrlicks reagent was tested due to the reaction of this reagent with furan rings[77] (Figure 12).[78] The reaction of

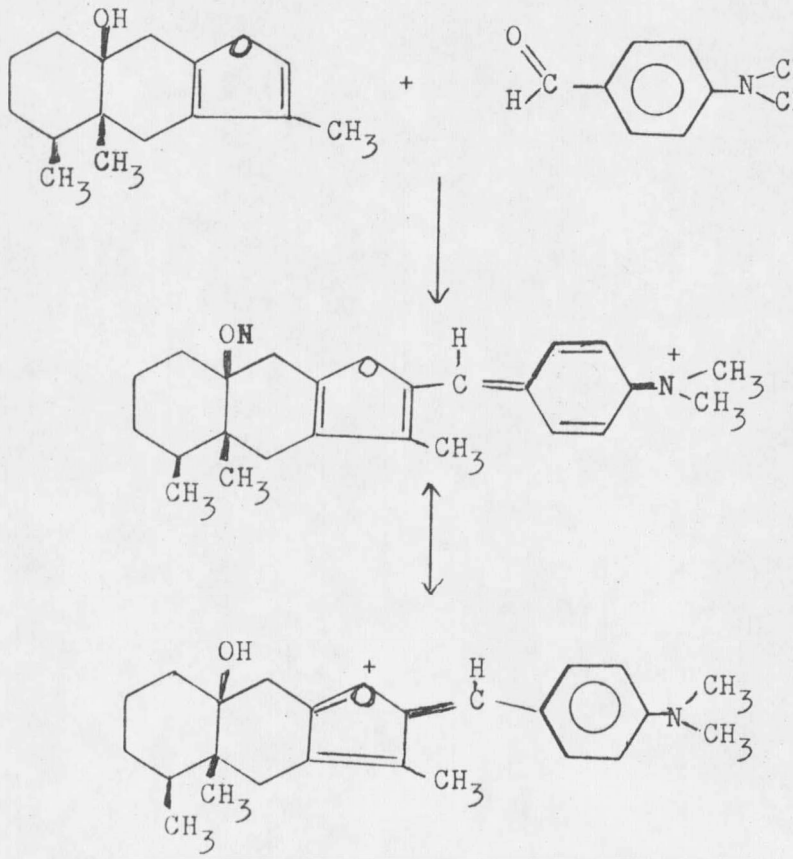


Figure 12. Reaction of tetradymol with Ehrlicks reagent[78]

tetradymol with this reagent was very sensitive to small concentrations of the toxin. With this reaction 2mg/ml of tetradymol could be measured in solution with an accuracy of ±0.5ug. This sensitivity would be beneficial when measuring levels of toxin found in animal organs after poisoning. After the accumulated data were run through the least squares program in the computer to obtain a standard curve, an equation of a line was obtained:

$$y = 66x - 4.7$$

y = concentration, x = O.D.

Ehrlicks reagent and this equation were used in all experiments where measurements of tetradymol concentration were necessary.

Having an accurate and sensitive method of quantitating tetradymol allowed research to proceed to substantiate the assumption that tetradymol was an hepatotoxin and to illucidate the fate of tetradymol in the system. After a compound is introduced to the animal system via the stomach, it is absorbed into the blood stream from the small intestine. The blood delivers the compound to the liver where it may act upon and be acted upon by various metabolizing systems. A lipid soluble compound such as tetradymol may be transformed or conjugated into a more water soluble form and will then be eliminated in the urine. Water soluble compounds do not

require transformation to be eliminated in the urine.

Early experiments indicated that tetradymol was relatively unstable with respect to light, heat, and acidic conditions. Since the stomach is rather acidic (pH 1.0-2.0) it was important to test the stability under those conditions. Results given in Table 9, proved tetradymol to be unstable in concentrated HCl but stable in 1M HCl, pH 1, and 0.01M phosphate buffer, pH 7.4. The pH of 1M HCl is close to the pH of the stomach. Thus experiments indicated that tetradymol would probably be stable in the stomach.

Table 9

Acid Lability of Tetradymol

Conditions	pH	% recovered
Conc. HCl	-1.08	0
1M HCl	1.00	96
0.01M Phosphate buffer	7.40	93

The next step was to test tetradymol directly in the stomach. The results of this experiment are shown in Table 10. Tetradymol was quantitatively recoverable over this time period, allowing for increasing absorption from the stomach and solubilizing into the stomach wall due to the lipophilicity of the compound. From these results it was concluded that tetradymol was eliminated from the stomach unchanged.

Table 10

Tetradymol Stability in Stomach

Time (min)	mg recovered	% recovered
0	0.98±0.014	98
15	0.95±0.046	95
30	0.93±0.070	93
45	0.78±0.006	78

Absorption from the small intestine would not be a problem since tetradymol was stable at pH 7.4 which is close to the pH in the small intestine (pH~7.0).

Tetradymol is a lipid-like compound and consequently should be lipid soluble. When studying lipid soluble compounds it is to be expected that the compound will be located throughout the body after administration and it will be fairly evenly distributed through the body.[79] The distribution of tetradymol was studied by poisoning mice with a lethal dose (350mg/kg) of tetradymol, sacrificing three mice every two hours, extracting various organs, and evaluating the toxin concentration. The organs examined were brain, heart, lung, stomach, kidney, pancreas, liver, and upper and lower intestine. This gave the concentration of tetradymol per organ over an eight hour time period. These results are shown in Table 11.

Table 11

Tetradymol Recovery from Organs

Time (hr)	Tetradymol recovered from: stomach *,o (%)	U.Int. *,o (%)	L.Int. *,o (%)	Liver *,o (%)	other organs *,o (%)	Total oo (%)
2	57.6	21.0	6.9	1.4	2.62	27.1
4	63.0	8.6	5.9	1.3	4.24	18.9
6	49.1	7.7	11.7	3.9	5.52	14.7
8	41.1	26.5	13.9	3.8	2.94	11.7

* % of total recovered
o corrected by subtracting furan level of control organs from level of poisoned organs
oo % recovered from total given

As can be seen from Table 11, tetradymol recovery went down over the eight hour period which would be expected due to increasing absorption into the tissue and biotransformation into metabolite(s). Since the toxin was given directly into the stomach, the highest level of tetradymol recovered was in this organ and decreases over the time period due to elimination from and absorption by the stomach. The remainder of the recovered toxin was located in all the organs examined and these organs had approximately the same amount of toxin present. Attempts were made to release the toxin from the tissues by treating the organs with various tissue solubilizers; this did not result in recovery of any more tetradymol. When

tetradymol was treated under the same conditions as the tissues had been, it could no longer be detected by the Ehrlicks reagent indicating the loss of the furan ring.

These results did not indicate that tetradymol directly affected any particular organ. However, it was noticed, on removal of the various organs from the animal, that the liver appeared discolored with yellow areas and was friable.

After being absorbed into the lipid layers lipid soluble compounds will slowly diffuse out, be metabolized, and continue to appear in the feces and urine of animals given such compounds.[79] A sublethal dose (100mg/kg) of tetradymol was given to the mice and the urine and feces were collected for seven twenty-four hour periods (Table 12.)

On the seventh day the toxin was not detected in the urine and feces and the experimented was terminated at this point. The amount recovered per day varied due to the food consumption, physical activity and eliminations of the mouse. Day one was the highest since the mouse would be eliminating whatever toxin had not been absorbed. This indicated that tetradymol could survive in the animal system for an extended period and that tetradymol was being solubilized by the tissues and was then slowly released.

Although it was not known that tetradymol survived the animal system and was distributed throughout the system, the

Table 12

Elimination of Tetradymol

Day	mg recovered*	% recovered°
1	.091±0.070	3.03
2	.028±0.006	0.93
3	.028±0.012	0.93
4	.006±0.005	0.20
5	.013±0.007	0.43
6	.016±0.005	0.53
7	0	0
Total	.182±0.015	6.10

*corrected by subtracting control urine and feces furan level from value after poisoning

°% recovered was based on total amount given

question remained as to which organ(s), if any, did tetradymol affect. The answer to this question was found in doing histology on the various organs that had been looked at previously in distribution. Mice were poisoned with a lethal dose (350mg/kg) of tetradymol and every two hours three were sacrificed, the organs removed, histology slides prepared and stained with hematoxylin-eosin. In viewing the resulting slides under a light microscope, it was found that only the liver reflected any damage and the other organs appeared essentially normal. The initial suggestion of hepatic damage was apparent after only two hours and was reflected in the

swelling of the cells in the centralobular area. Swelling was uniform and accompanied by some slight, fine cytoplasmic vacoular degeneration. Figures 13, 14, 15, and 16 are liver slides taken over a four to ten hour time period. In these figures it can be seen that hepatic damage became more severe as time progressed. Figure 13 shows a uniform, mild cytoplasmic vacoular degeneration which spreads through the central region and into the midzonal area. Six hours after poisoning, Figure 14, the cytoplasmic vacoular degeneration is more severe, the nuclei are becoming irregular, and some nuclei are missing. After eight hours, Figure 15, the centralobular degeneration is severe and necrosis is apparent. The nuclei are small in the damaged area indicating pyknosis and dilated blood filled sinusoids are present. Glycogen depletion is apparent as seen by the large holes in the tissue where the glycogen has left. Further support for glycogen depletion was obtained by doing a periodic acid-shift stain on liver slices over the same time period. This stain is specific for glycogen. At the various time periods the stain became lighter indicating a loss of glycogen. After ten hours, Figure 16, the necrosis is very severe. Kayloysis, pyknosis, and karyorrhexis are apparent. There is a loss of cells and pools of red blood cells are present. Glycogen depletion is more apparent at this time. The mice used for the eight and ten hour slides were on