



Paradoxical uncoupling activity of tetradymol isolated from *Tetradymia glabrata*
by Andrij Holian

A thesis submitted in partial fulfillment . of the requirements for the degree of DOCTOR OF
PHILOSOPHY in Chemistry
Montana State University
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Abstract:

Clinical studies on acute toxicity of tetradymol have substantiated previous research in the hepatotoxic activity of tetradymol. Furthermore, studies herein suggest that tetradymol is modifying energy metabolism, "In vitro" studies on whole mitochondria have established tetradymol as an uncoupler of oxidative phosphorylation. Using submitochondrial particles, tetradymol behaved as an ATPase inhibitor. This paradoxical behavior has been used to suggest that tetradymol does not cross over either side of the mitochondrial membrane.

Using both mitochondria and submitochondrial particles, related compounds have been investigated with respect to various parameters of coupled respiration. With the knowledge gained, a stereochemical model of the active site has been proposed.

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Chemistry

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TABLE OF CONTENTS

	page
LIST OF FIGURES	v
LIST OF TABLES	vi
ABSTRACT	ix
INTRODUCTION	1
RESULTS AND DISCUSSION	29
Conclusion	61
EXPERIMENTAL SECTION	65
Reagents	65
Instruments	68
Mice	68
Histology slides	69
Isolation of mitochondria and submitochondrial particles	69
Standard determinations	69
Experiments	71
APPENDIX	
A. Clinical tests	94
B. Structures of Related Compounds	96
LITERATURE CITED	99

LIST OF FIGURES

	page
1. Urea cycle	6
2. Representation of mitochondrion	7
3. Representation of cristae	8
4. Representation of submitochondrial particle	9
5. Electron chain and oxidative phosphorylation	10
6. State 4 respiration vs uncoupler concentration	17
7. Rifampicin and its three metabolites	21
8. Structure of chlordimeform	22
9. p-nitro analog of chlordimeform	23
10. Structure of clofibrate	24
11. Dependence on K^+ concentration	28
12. Effect of tetradymol on state 4 respiration	34
13. Effect of tetradymol on RC and P/O	36
14. Tetradymol uncoupling vs pH	48
15. Tetradymol uncoupling vs protein	51

LIST OF TABLES

	page
1. Relationship of histology to clinical results	4
2. Inhibitors of the mitochondrial ETS	12
3. Various classifications of uncouplers	13
4. Generalized uncoupler characteristics	14
5. Clinical chemistry	29
6. Citrulline production	33
7. Effect of tetradymol on state 4 respiration	35
8. Effect of tetradymol on RC	37
9. Effect of tetradymol on P/O ratios	38
10. Effect of tetradymol on P/O ratios	39
11. Relative uncoupling at 3 sites	40
12a. Reversal of ATPase inhibition	41
12b. Reversal of ATPase inhibition	41
13. Mitochondrial ATPase activity	42
14. Reverse electron flow in mitochondria	42
15. Effect of tetradymol on mitochondria	45
16. Comparison of SDS and tetradymol uncoupling	45
17. Varying K^+ concentration	46
18. Tetradymol uncoupling vs pH	47
19. Tetradymol uncoupling vs protein	49
20a. Effect of BSA	50
20b. Effect of BSA	50
21. Reverse electron flow in submitochondrial particles	53

	page
21. Reverse electron flow in submitochondrial particles	53
22. Submitochondrial ATPase activity	54
23. Effect of tetradymol on submitochondrial oxygen consumption rates	55
24. P/O in submitochondrial particles	56
25. Submitochondrial ATPase activity	57
26. Mitochondrial ATPase activity	58
27. Relative uncoupling ability of tetradymol-like compounds	60
28. Ornithine transcarbamylase activity	73
29. Urea production	73
30. Effect of tetradymol on mitochondrial oxidative phosphorylation	76
31. Mitochondrial P/O	77
32. Mitochondrial reverse electron flow	78
33. Submitochondrial reverse electron flow	79
34. Mitochondrial ATPase	80
35. Submitochondrial ATPase	81
36. Reversal by tetradymol of DCCI inhibited mitochondrial ATPase	82
37. Effect of BSA on tetradymol uncoupling	83
38. Effect of varying K^+ on tetradymol uncoupling	84
39. Effect of varying pH on tetradymol uncoupling	85
40. Effect of varying mitochondrial protein	86
41. Effects on O.D. of a mitochondrial solution	87
42. SDS effect on state 4	87

- | | |
|--|----|
| 43. Effect of tetradymol on O_2 uptake with submitochondrial particles | 87 |
| 44. Uncoupling ability of tetradymol like compounds | 89 |
| 45. Relative uncoupling at 3 sites | 91 |

Abstract

Clinical studies on acute toxicity of tetradymol have substantiated previous research in the hepatotoxic activity of tetradymol. Furthermore, studies herein suggest that tetradymol is modifying energy metabolism.

"In vitro" studies on whole mitochondria have established tetradymol as an uncoupler of oxidative phosphorylation. Using submitochondrial particles, tetradymol behaved as an ATPase inhibitor. This paradoxical behavior has been used to suggest that tetradymol does not cross over either side of the mitochondrial membrane.

Using both mitochondria and submitochondrial particles, related compounds have been investigated with respect to various parameters of coupled respiration. With the knowledge gained, a stereochemical model of the active site has been proposed.

INTRODUCTION

This study has been directed towards obtaining information as to the toxicity of tetradymol. For a better understanding of the research and implications involved, a number of pertinent subjects will be reviewed and references given for additional information. Initially a brief review of previous work on the project will be presented. For more indepth information the reader is referred to both S. Holian¹ and S. Reeder's² theses.

Tetradymol is a toxic substance isolated from the plant Tetradymia glabrata. Reeder² observed from whole plant feeding experiments with sheep that: 1) feeding 1% of body weight for three days resulted in death; 2) bromsulphalein (BSP) clearance time was greatly lengthened; 3) blood serum ammonia levels were elevated three to six fold in poisoned sheep. The latter two observations led to the conclusion of liver dysfunction.

Autopsies were performed on all sheep that were poisoned. The results are summarized below:³

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 principles were manifested primarily in the liver.

Information obtained from histology studies on mice after being fed purified tetradymol established that only the liver was affected. Tetradymol causes dose dependent centralobular necrosis that is apparent at two hours by swelling of the liver cells and increases in severity up to death (10 hours). The damage can be seen as cytoplasmic vacuolar degeneration, necrosis (complete loss of cell differentiation), and nuclear pyknosis (shrinking of nuclei) and karyorrhexis (swelling and disruption of nuclei).⁴

In addition, it was established that metabolite(s) formed from the metabolism of tetradymol by the liver mixed function oxidase system was more toxic than tetradymol.⁵ Furthermore, it was proposed that tetradymol itself could still be toxic.

To gain further general information on the toxicity of tetradymol a number of clinical serum tests were conducted. The purpose of these tests was to gain additional information concerning organ involvement and to observe altered serum metabolite concentrations that might implicate a possible mode of action of tetradymol "in vivo". A clarification and possible interpretations of those tests that will be important in the discussion will follow. More detail on these following tests and others can be found in the following references.^{6,7,8}

A test related to the BSP clearance time (mentioned earlier) is determination of bilirubin levels. In both cases (BSP and bilirubin) the liver extracts the compounds and excretes them in the bile. Additionally, bilirubin is conjugated before it is excreted.⁹

Two kinds of bilirubin are measured. One is termed direct reacting bilirubin which has been conjugated in the liver with glucuronide. Increases occur commonly in cases of obstructive jaundice, intrahepatic obstruction or hepatocellular damage. Hepatotoxic agents, such as, erythromycin or chlorpromazine, will cause elevations due to intrahepatic obstruction and damage.¹⁰ The other form of bilirubin (which is of greater interest in this research) is termed indirect reacting (unconjugated) bilirubin. This form is bound to serum albumin until extracted by the liver to be conjugated. Elevation of this form is due to either increases of hemolytic anemia or from deficiencies in the liver conjugation processes. An agent, such as, methyltestosterone, will cause this type of damage.¹⁰

The clinical manifestations of liver injury can be related to the histological observations (Table 1).¹⁰ Since tetradymol has been shown (stated earlier) to cause a zonal necrosis, clinical tests could be used to substantiate these findings.

Liver necrosis will produce high levels of serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT). Slight increases of alkaline phosphatase and variable levels of flocculation tests are dependent on different types of necrosis. Jaundice has lower transaminase levels and flocculation tests are low. Cholestasis with accompanied portal inflammation gives high alkaline phosphatase levels; while, without inflammation alkaline phosphatase levels are normal. Agents, such as, sulfonamide will produce various combinations of the above by causing both cytotoxic necrosis and cholestasis.

Table 1
Relationship of Histology to Clinical Results¹⁰

HISTOLOGIC LESION		BIOCHEMICAL ABNORMALITIES			
		SGOT AND SGPT	ALK. PHOS.	THYMOL TURB. AND CEPH. FLOCC.	EXAMPLES OF HEPATOTOXIC AGENTS
I. Cytotoxic Necrosis—Zonal		↑↑↑↑	↑	±	CCl ₄
	Necrosis—Diffuse	↑↑↑↑	↑	↑↑	Iproniazid
	Fatty Metamorphosis	↑↑	±	±	Tetracycline
II. Cholestatic					
A.	With Portal Inflammation	↑↑	↑↑↑↑	—	Erythromycin; Chlorpromazine
B.	Without portal inflammation (Bland)	↑↑	±	—	Methyltest.
III. Mixed	Mixture of Cytotoxic and Cholestatic	↑ → ↑↑↑↑	↑ → ↑↑↑↑	→ ↑↑	Sulfonamides

Measurement of blood ammonia levels is not a routine clinical test but elevations in blood ammonia have been implicated in a number of different liver dysfunctions; e.g., hepatic coma,^{11,12} Reye's Syndrome,^{13,14} and alcoholic cirrhosis.¹⁵ Hepatic coma can accompany various forms of liver dysfunction as a complication. The exact cause of the coma is not certain but accompanied increases in blood ammonia levels have been suspected. The recently important Reye's Syndrome has, as a symptom, elevated blood ammonia levels. Administration of ammonium salts has been shown to increase blood ammonia and cause death.^{16,17} The cause for the rise in blood ammonia can be due to a number of factors where the urea cycle is concerned. In cases of ammonium salt poisoning, the activity of the urea cycle¹⁸ (figure 1) is not sufficient to assimilate the ammonia into urea. Otherwise some deficiency in the urea cycle could be causing poor assimilation of ammonia. Genetic deficiencies in the urea cycle enzymes have been reported to cause increases in blood ammonia.^{19,20} Lack of ATP has also been suggested as a contributing factor since three ATP's (four high energy bonds) are required for each urea synthesized.²¹

There are a number of methods that have been tried (sometimes successfully) for reducing blood ammonia levels. These methods can be divided into two groups: 1) Administration of glutamic acid with the hope that ammonia will be removed by

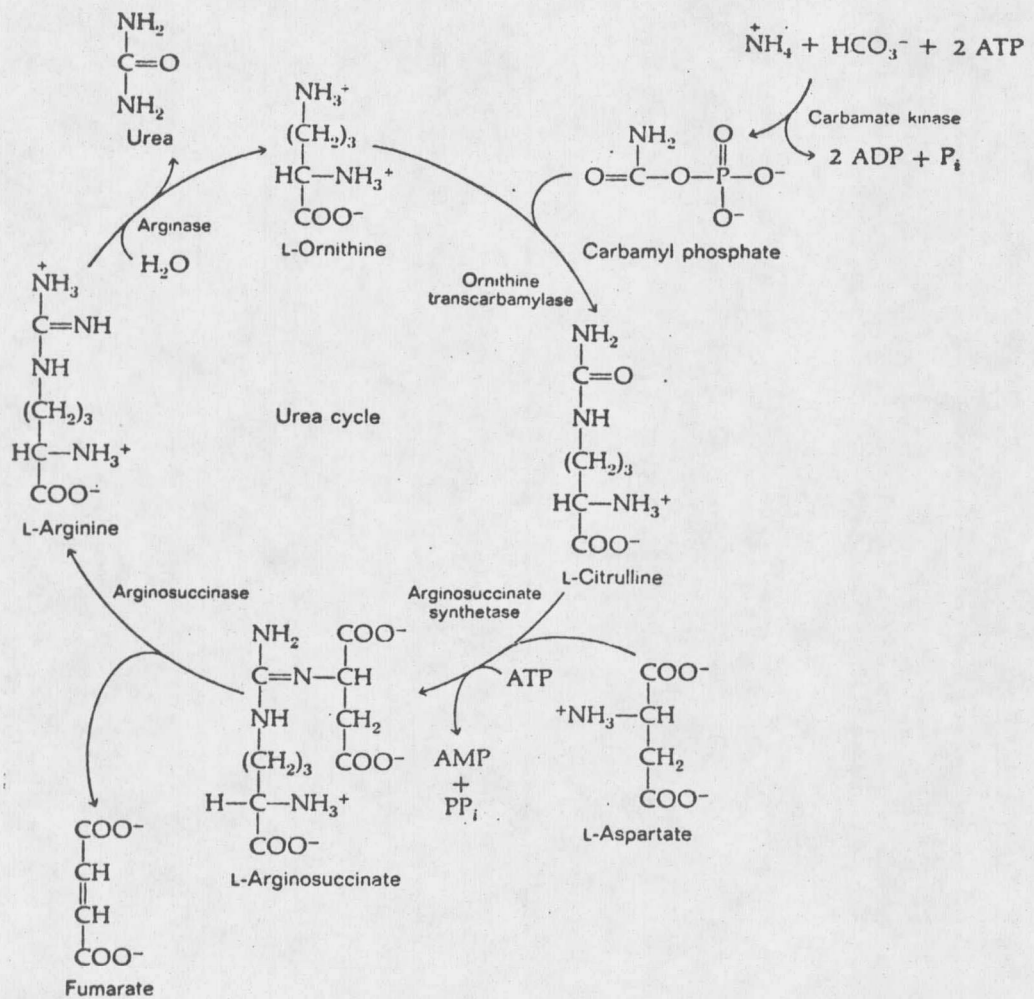


Figure 1: Urea cycle

formation of glutamine¹² or 2) administration of urea cycle amino acids with the hope of stimulating the cycle.^{12,22,23,24,25} Using the first method, results are short lived since glutaminase will cleave glutamine back into glutamic acid and ammonia.²⁶ Both methods demonstrated prevention of death from ammonium salts.²² In actual clinical cases some successes have been reported^{24,25} but in general, since the symptom is treated instead of the problem, blood ammonia rises to previous or higher levels.²⁷

Earlier, reduction of ATP levels was cited as a possible cause for elevated blood ammonia levels. Part of the urea cycle and most of the ATP production takes place in the mitochondria. Below is a crosssectional diagram of a liver mitochondrion (figure 2).²⁸

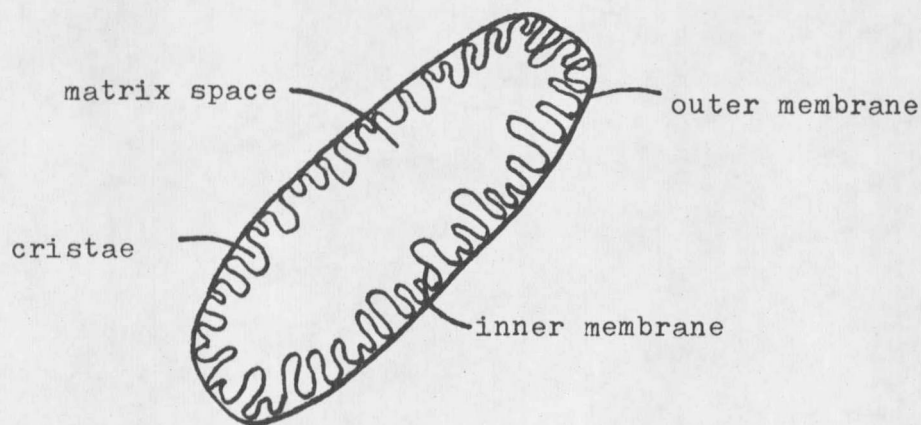


Figure 2: Representation of mitochondrion

The outer membrane is fairly permeable permitting translocation of such things as sucrose, other simple sugars, and most other solutes of molecular weight less than 5000 daltons.²⁹ Located on the outer membrane are certain characteristic enzymes such as monoamine oxidase, fatty acid thiokinase, kynurenina hydroxylase and rotenone-insensitive cytochrome c reductase.³⁰ Between the membranes, adenylate kinase and nucleoside diphosphokinase are located.³⁰ Found as a part of the inner membrane are enzymes responsible for electron transport system (ETS) and oxidative phosphorylation.³⁰ The inner membrane is permeable to small neutral molecules, the undissociated forms of some monocarboxylic acids (such as, β -hydroxybutyric acid and acetoacetic acid), and to ammonia. However, it is impermeable to H^+ , OH^- , K^+ , Cl^- , and most small ions.²⁹ In the matrix space most of the TCA cycle enzymes along with glutamate dehydrogenase and fatty acid oxidation enzymes can be found.³⁰

Located on the inside of the inner membrane are stalk-like structures associated with the oxidative phosphorylation enzymes (figure 3).³¹ When mitochondria are sonicated in a



Figure 3: Representation of cristae

hypotonic medium the mitochondria rupture and the inner membrane reforms in an inverted form (figure 4).³¹ During sonication

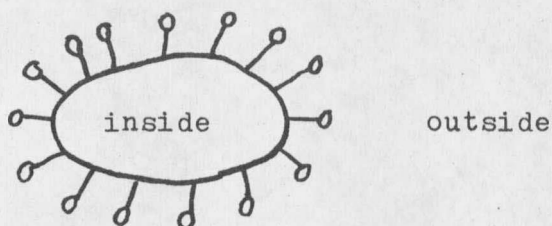


Figure 4: Representation of submitochondrial particle

the cristae become pinched off to form the submitochondrial particles. Both the mitochondria and submitochondrial particles retain electron transport and oxidative phosphorylation abilities.³² Various representations of the arrangement between enzymes in the electron transport chain and oxidative phosphorylation have been presented; in figure 5 is one representation.^{33,34}

Hanstein³⁵ has been able to obtain five complexes from mitochondria that are part of the ETS and oxidative phosphorylation. Four of these complexes comprise the ETS and are the traditional ones obtained by other workers.³⁶ The fifth complex contains the oxidative phosphorylation enzymes. A good discussion of the first four complexes can be found elsewhere.³⁷ The fifth complex contains F_1 which is the ATPase enzymes, F_0 which is the oligomycin sensitive conferring protein (also sensi-

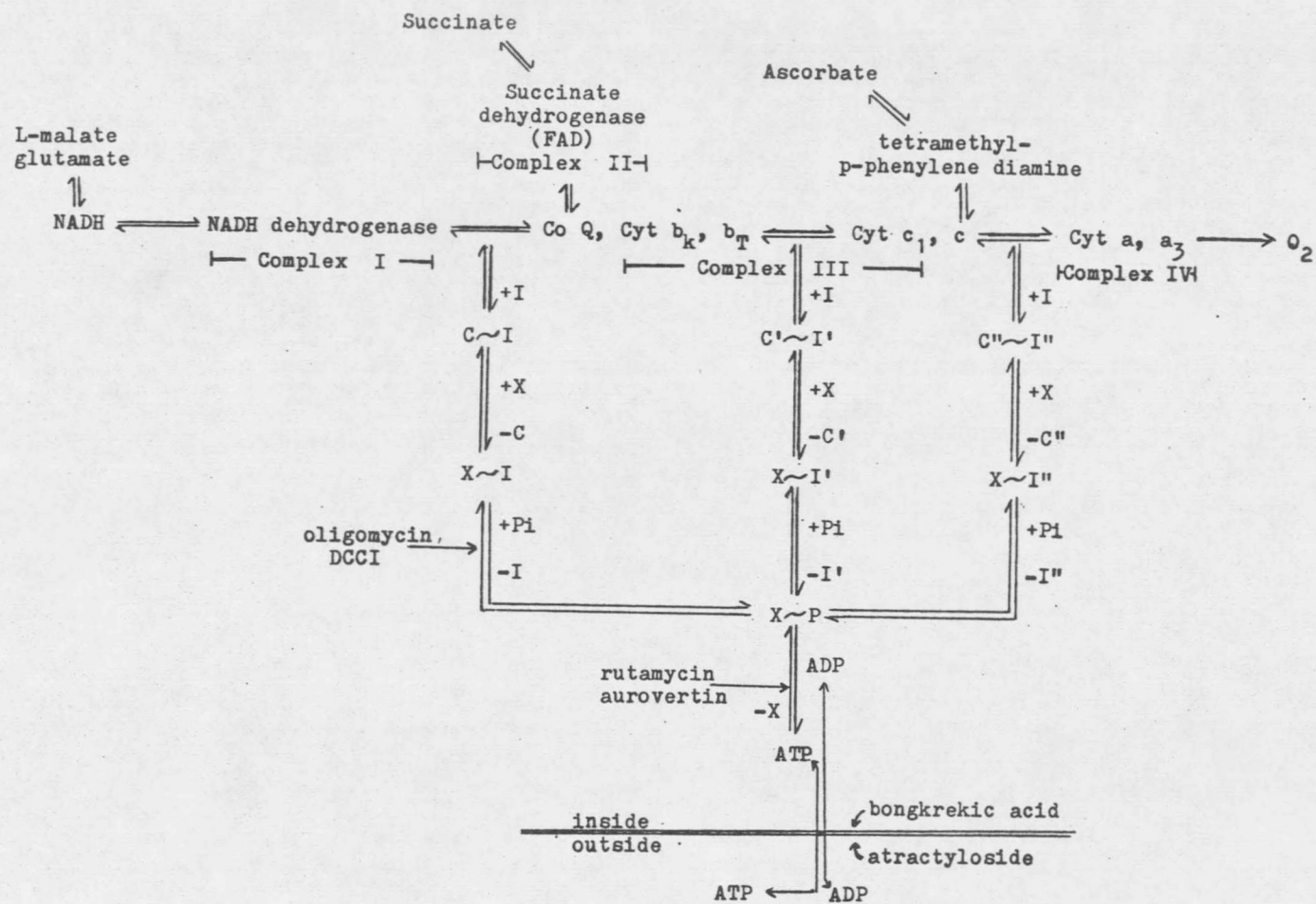


Figure 5: Electron chain and oxidative phosphorylation

tive to dicyclohexylcarbodiimide (DCCI)), and a protein or series of proteins that bind uncouplers. Activity can be restored upon recombination of all five complexes. Further discussion of uncoupler binding will follow later. For a review of the structure of ATPase containing F_1 and F_0 refer to Senior.³⁸

As will be illustrated in the discussion, tetradymol affects oxidative phosphorylation. Tetradymol is an uncoupler of oxidative phosphorylation in whole mitochondria and an inhibitor of ATPase in submitochondrial particles. The following discussion will deal with various aspects of compounds that can affect oxidative phosphorylation and the ETS. Four groups of compounds will be considered: 1) ETS inhibitors; 2) uncouplers; 3) ATPase inhibitors and 4) ionophores.

Electron transport inhibitors are compounds that act at a specific site in the ETS causing blockage of electron flow past that point. A list of some traditional ETS inhibitors and a few recent findings is presented in Table 2.

The effects of these compounds will manifest themselves by a reduction of oxygen consumption.³⁹ This reduction will not be reversed by uncouplers. No effect will be observed on ATPase activity. If an ETS inhibitor acts at complex I (pg. 10) the reverse electron flow will be inhibited. Otherwise, if the ETS inhibitor acts at either complex II, III, or IV no effect will be observed. Observations of ATPase activity, reversal of

inhibition by uncouplers and reverse electron flow will be described in the following section dealing with uncouplers.

Table 2

Inhibitors of the Mitochondrial Electron Transport Chain

<u>Compound</u>	<u>Inhibitory site</u>	<u>Ref</u>
Dibromothymoquinone	Co Q	39
Hydroxy-substituted-1,4-quinones	b $\rightarrow$ C ₁	40
Funiculosin	Co Q $\rightarrow$ Cyt C	41
Rotenone, Piericidin, Amytal, Seconal	FMN $\rightarrow$ Co Q	42
Thenoyltrifluoroacetone	peptide $\rightarrow$ FAD $\rightarrow$ Co Q	42
Antimycin A	Cyt b $\rightarrow$ Cyt c ₁	42
HCN, HN ₃ , CO	Cyt c $\rightarrow$ O ₂	42

In discussing uncoupler action the effects of ATPase inhibitors are involved. Therefore, the two subject areas will be intermixed followed by a brief discussion on the classes of ATPase inhibitors.

Uncouplers are compounds that stimulate respiration and allow ETS to function without concomittant ATP synthesis or other work except heat production. A list of classes of compounds that are uncouplers is given in Table 3.⁴³ These compounds have the ability by uncoupling to exert their effects in a number of different measurable ways. A list of characteristics

Table 3

Various Classifications of Uncouplers⁴³

<u>Class</u>	<u>Example</u>
Antibiotics	Gramicidin
Barbiturates	Thiopental: 5-ethyl-5-(1-methyl-butyl)-2-thiobarbituric acid
Benzimidazoles	4,5,6,7-Tetrachloro-2-trifluoro-methylbenzimidazole
Bile pigments	Bilirubin
Carbonyl cyanide phenylhydrazones	Carbonyl cyanide m-chlorophenyl-hydrazone
Coumarins	Dicoumarol: 3,3'-methylenebis(4-hydroxycoumarin)
Halophenols	Pentochlorophenol
Hormones	Thyroxine
Nitrophenols	2,4-Dinitrophenol
Phenothiazines	Chlorpromazine: 2-chloro-10(3-dimethylaminopropyl)-phenothiazine
Quinacrines	Atabrine: 3-chloro-7-methoxy-9-(1-methyl-4-diethylamino-butyl-amino) acridine
Phlorobutyrophenones	Desaspidin
Salicylanilides	Yomesan: 2',5-dichloro-4'-nitro-salicylanilide
Salicylates	Salicylic acid
Unsaturated fatty acids	Oleic acid

describing these effects and their general physical characteristics has been compiled (Table 4).⁴⁴

Table 4

Generalized Uncoupler Characteristics⁴⁴

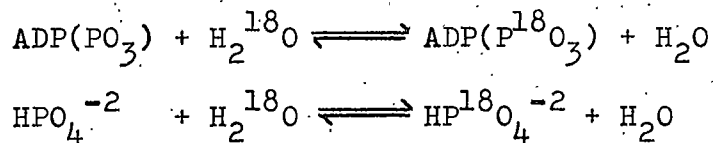
1. Stimulate state 4 respiration
2. Abolish respiratory chain-linked phosphorylation
3. Can increase respiration in particles inhibited by ATPase inhibitors
4. Stimulate latent ATPase and inhibit reverse electron flow in both mitochondria and sub-mitochondrial particles
5. Are usually highly lipid soluble weak acids (or anions of such acids), pKa between 3 to 9⁴⁷ or 4 to 6⁴³

Uncouplers stimulate state 4 respiration which occurs in the presence of substrate (such as, glutamate, succinate, TMPD with ascorbate) and inorganic phosphate and in the absence of a terminal phosphate acceptor. State 3 respiration, unaffected by uncouplers, occurs in the presence of substrate and inorganic phosphate along with a terminal phosphate acceptor, usually ADP. The ratio of state 3 to state 4 rates is referred to as respiratory control (RC).⁴⁵ An uncoupler usually causes a loss in RC. A decrease in the ADP to oxygen consumption or P/O ratio would be observed with abolished respiratory chain-linked phosphorylation. According to Racker

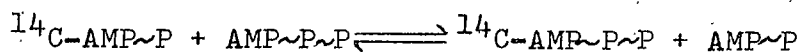
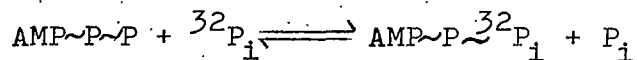
a compound that causes an increase in both RC and P/O makes that compound a true uncoupler.⁴⁶ An ATPase inhibitor, such as oligomycin, will cause complete reduction of state 3 respiration. Adding an uncoupler should stimulate the respiration that has been inhibited without phosphorylation taking place.

ATPase causes the hydrolysis of ATP which is usually measured as amount of P_i liberated.⁴⁸ Activity of this enzyme is usually stimulated by uncouplers and that stimulation is inhibited by ATPase inhibitors such as, oligomycin, the converse is also true. Reverse electron flow is accomplished by the addition of succinate as the substrate, ATP as the energy source and NAD^+ as the terminal electron and proton acceptor.⁴⁹ As the name implies this activity is the reverse of what normally occurs. This reaction can be inhibited by uncouplers, ATPase inhibitors or electron transport inhibitors (as described on pg. 11).⁴⁹

Other than those characteristics listed, uncouplers affect other mitochondrial processes. One of these is the ATPase exchange reactions. Two of the exchange reactions are very rapid:⁵⁰



and two are slower:



These exchange reactions are a part of oxidative phosphorylation and take place in F_1 . All four of these exchange reactions are inhibited by uncouplers and ATPase inhibitors.⁵⁰

Some of the mitochondrial transport processes are linked to the electron transport chain. By virtue of not allowing work production from the ETS those mitochondrial transport processes linked to the ETS are inhibited by uncouplers.

Presently a number of these transport systems are being investigated by other workers. Mitochondrial uptake of acetate, malate and phosphate has been shown to be inhibited by uncouplers such as the carbonyl cyanides.^{51,52} The exchange of ADP in for ATP out of the mitochondria has also been linked to the ETS and is susceptible to uncouplers.⁵³ La Noue⁵³ has recently established that aspartate efflux is inhibited by carbonyl cyanides.

One of the urea cycle amino acids, ornithine, has to go from the cytosol, where it is formed, into the mitochondria to be condensed with carbamyl phosphate. This transport process has been linked to the ETS and is inhibited by uncouplers.²⁸

Uncoupler related processes such as stimulation of both state 4 and ATPase activity are sensitive to high concentrations

of uncouplers. It has been established that both processes are inhibited at high concentrations of all uncouplers studied.^{54,55} Plots of uncoupler concentration vs state 4 respiration will generally have a curve of the following nature (Figure 6).⁵⁴ Uncouplers will increase the control state 4 activity but at high concentrations inhibit all respiratory activity.

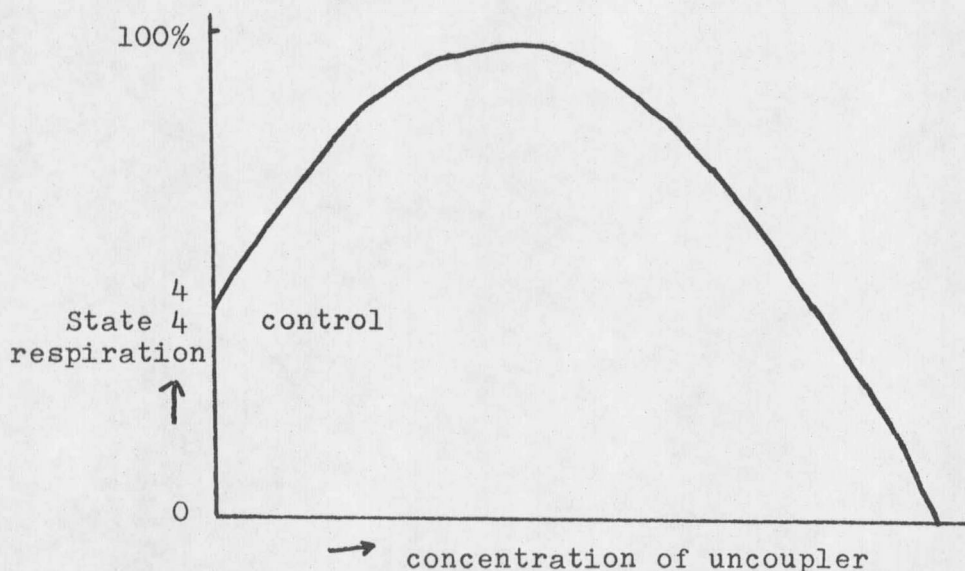


Figure 6: Activity of state 4 respiration vs uncoupler concentration

Uncouplers such as 2,4-DNP, pentachlorophenol, dicoumarol, and m-chlorocarbonyl cyanide phenyl hydrazone, are considered to be about equally effective at all three phosphorylation sites.⁵⁶ However, Katyare, et al,⁵⁷ have shown that 2,4-DNP is virtually ineffective at the second phosphorylation step. More information will be presented later that show some new uncouplers may

not act equally at all three sites.

The mechanism of action of uncouplers is lost in the complexity of oxidative phosphorylation. Often times one mechanism is used to try to explain the other. The most generalized agreement of action is that uncouplers function as proton carriers since the pKa's of most of the classical uncouplers are relatively close to neutrality (pKa 3-9). Exactly how they function as a proton carrier is unclear. For a detailed description of the theories involved the reader is referred elsewhere.^{58,59,60,61}

As stated earlier (pg. 9) it is proposed that uncouplers bind to specific proteins that are a part of complex five. Of the five complexes isolated only the fifth complex, containing ATPase, was found to contain any specific uncoupler binding. Work by Hanstein and Hatefi⁶² has shown 2-azido-4-nitrophenol binds competitively with 2,4-DNP, PCP, mCCCP, and 5-chloro-3-t-butyl-2'-chloro-4'-nitrosalicylanilide. They implied that these uncoupler binding sites are the same sites that cause uncoupling. The activity of 2-azido-4-nitrophenol was found to be unaffected by ATPase inhibitors, ETS inhibitors, or ionophores. Bakker, et al,⁶³ present contradictory evidence concerning uncoupler binding. They show that a water soluble uncoupler like 2,4-DNP does not bind to mitochondria, therefore it could not be binding to any uncoupler binding sites in the mitochondria.

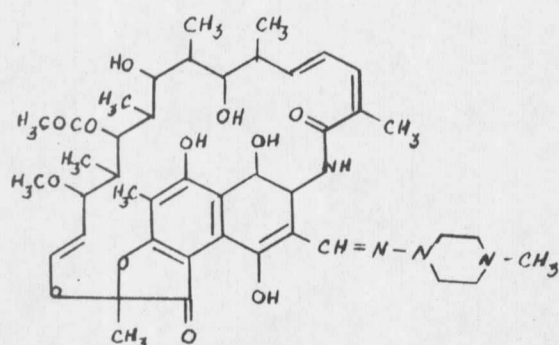
Efforts have been directed toward establishing steric requirements of uncouplers and binding sites; and whether, changes in lipid solubility or pKa's have any effect on uncoupler activity. Using a series of phenolic compounds Hemker⁶⁴ observed that increasing the lipophilicity increased uncoupling ability, whereas, increasing the size of alkyl substituents in 4-substituted 2,6-dinitrophenols had no effect on uncoupling ability. The work of Terada and Muraoka⁶⁵ using 3'-substituted analogues of N-phenylanthranilic acid also has shown that increasing lipid solubility increased uncoupling ability, while increasing the pKa from 4 to 7 also increased uncoupling ability. Tollenaere⁶⁶ studied three groups of uncouplers, substituted salicylanilides, 2-trifluoromethylbenzimidazols, and phenols. The data were correlated by means of the Hansch free-energy relationships involving the lipophilicity and electronic substituent constants. Steric effects seemed to play a minor role. It was suggested that the phenolic moiety adjacent to the amide function may be partly responsible for the high uncoupling activity of the salicylanilides. Contradictory evidence was presented by Story, et al,⁶⁷ concerning the effect of steric requirements. Using 4'-substituted 3,5-dichlorosalicylanilides it was determined that uncoupling activity was dependent on the size of the substituents. With the compounds they tested increasing the size of the substituent increased the activity.

Thus one can conclude both, lipophilicity and steric effects, play a role in uncoupling activity.

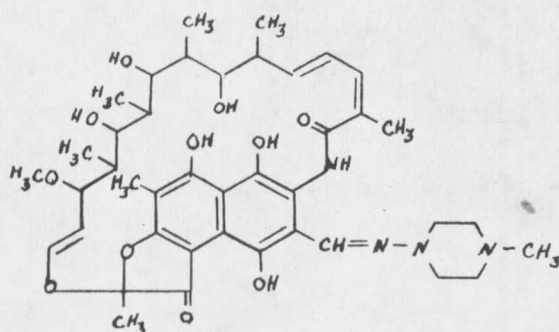
It has been observed that defatted bovine serum albumin (BSA) has the ability to readily bind most uncouplers.⁵³ By binding the uncouplers and thereby preventing the uncoupler from interacting with the mitochondria, the effects are lost and mitochondrial respiration is returned to normal.

New uncoupling compounds are appearing frequently in the literature. Since tetradymol will be discussed as a unique uncoupler, some new compounds that are also unique in one fashion or another from the classical uncoupler descriptions will be discussed.

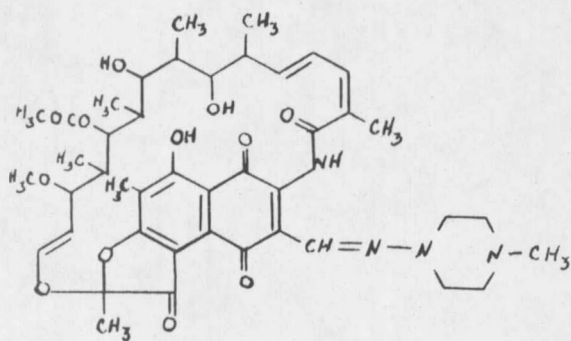
Rifampicin is a derivative of rifamycins isolated from S. mediterranei.⁶⁸ It is used as an antibiotic against both gram-positive and gram-negative bacteria. Metabolism of rifampicin has been shown to produce three main metabolites found in the urine (figure 7).⁶⁹ Rifampicin and its three metabolites were examined to observe any effect on respiration and several energy transfer reactions in rat liver mitochondria. Only one of the four compounds, 3-formyl rifamycin (3F-RFM-SV), exhibited activity. No conclusions concerning the lack of activity of the other three compounds were made (although the only consistent difference is the absence of $-\text{CH}=\text{N}-\text{N} \begin{array}{c} \diagup \quad \diagdown \\ \diagdown \quad \diagup \end{array} \text{N}-\text{CH}_3$ from 3F-RFM-SV) other than at the concentrations studied only



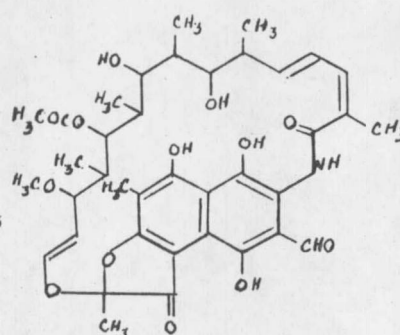
Rifampicin



25-Desacetylrifampicin



Rifampicin quinone



3-Formyl rifamycin SV

Figure 7: Rifampicin and its three metabolites

3F-RFM-SV had any uncoupling activity.

Chlordimeform is a novel acaricide, ovicide, and larvicide.⁷⁰ The oral LD₅₀ to male rats is 162-170mg/kg. It is lipid soluble and is reported to have a pKa of 6.8 (water/methanol, 1:1 v/v). At a concentration of 0.04μmoles/mg protein chlordimeform uncouples 50% of respiratory-chain phosphorylation. The uncoupling activity depends on mitochondrial protein concentration and can be reversed by the addition of BSA. ATPase inhibition by oligomycin is reversed by chlordimeform while the increased ATPase activity induced by chlordimeform was inhibited by oligomycin. The p-nitro analog of chlordimeform though is virtually devoid of uncoupling activity. The lack of uncoupling ability was attributed by the authors to decreased lipid solubility.

When 2,4-DNP was applied jointly with a concentration of chlordimeform, which represented 60-70% of the concentration

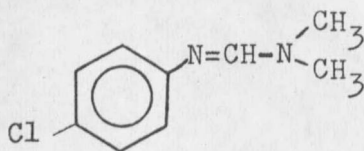


Figure 8: Structure of chlordimeform

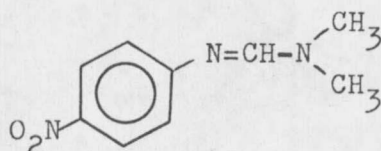


Figure 9: Structure of the p-nitro analog of chlordimeform

having maximal effectiveness on the parameters of coupled respiration, a clearly additive effect was observed on these parameters. The joint application of uncouplers which were lipid soluble bases with chlordimeform at the same concentration gave an antagonistic response. The conclusion Ado-Khatua and Hollingworth reached was that chlordimeform and 2,4-DNP acted at different sites in the mitochondria; whereas, earlier it was stated (pg. 18) that Hanstein and Hatefi⁶² observed competitive binding among various uncouplers at all sites.

Hanstein and Hatefi⁷¹ have studied a "unique" uncoupler of oxidative phosphorylation, viz. picrate (trinitrophenol). Concentrations of picrate up to 2mM had no effect on state 4 respiration or P/O ratios in either whole mitochondria or mitochondria depleted of their outer membranes (digitonin particles) using β -hydroxybutyrate as the substrate. Using submitochondrial particles, 93 μ M picrate caused 50% loss in the

P/O ratio using succinate as the substrate. The implication was that picrate was impermeable to the mitochondrial inner membrane and the uncoupler binding sites were on the inside. Using their uncoupler 2-azido-4-nitrophenol (NPA) (described previously) they observed competitive binding using submitochondrial particles. Since picrate probably did not cross the inner membrane they felt this was strong evidence that it was not uncoupling by being a proton carrier across the inner membrane.

The following compound is of interest for two reasons. On one hand the compound is a unique uncoupler which has two opposing actions in mitochondria. On the other hand two groups have generated two conflicting sets of data using clofibrate as an uncoupler (Panini and Kurup⁷² and Mackerer and Haettinger⁷³).

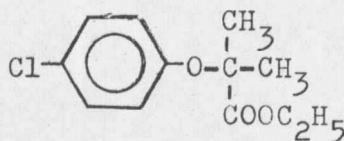


Figure 10: Structure of clofibrate
(chlorophenoxyisobutyrate ethyl ester)

Clofibrate is now being extensively used as an antihypercholesterolaemic drug. Toxicity has not been reported, but adverse changes, in the liver, have been. These changes are an increase in liver size and an increase of cellular content of peroxisomes and mitochondria.

Panini has determined that chlorophenoxyisobutyrate⁻Na⁺ (CPB) (at concentrations 4 μ moles/mg of mitochondrial protein) stimulates state 4 respiration, abolishes RC, reduces P/O at all three phosphorylation sites, and stimulates ATPase activity in mitochondria. ATPase activity was maximal at a concentration of 10 to 12 μ moles/mg protein then activity decreased at higher concentrations of CPB. Reverse electron flow in submitochondrial particles was also inhibited by CPB. These results are common and not unexpected for an uncoupler (as described earlier).

Panini and Kurup contend that unusual activity was demonstrated by CPB's ability to inhibit 2,4-DNP (200 μ M) stimulated ATPase activity in both mitochondria and submitochondrial particles. Furthermore, CPB at concentrations >4.0 μ moles/mg protein inhibited respiration in mitochondria. It was concluded from this that CPB belongs to a rare class of compounds termed "inhibitory uncouplers" by Racker.⁷⁴

Conflict between the two groups, Panini, et. al., and Mackerer, et. al., is apparent when the activity of CPB at the three phosphorylation sites is examined. Panini reports that

coupled phosphorylation at the first site was more sensitive than site three which was more sensitive than site two (3.0, 5.0, and 10.0 μ moles/mg protein, respectively). Mackerer, however, determined that site one was completely resistant to uncoupling action. At a concentration of 0.2 μ mole/mg protein, site two showed a 43% loss and site three a 72% loss of coupling activity.

Throughout, this discussion on uncouplers, ATPase inhibitors were mentioned several times. Three groups of ATPase inhibitors exist. In the first group there are two true inhibitors of ATPase, oligomycin and DCCI.⁷⁵ A second group of ATPase inhibitors, rutamycin and aurovertin, appear to block a later step than oligomycin and DCCI.⁷⁵ Oligomycin sensitive conferring protein is necessary to demonstrate either oligomycin or DCCI inhibition of ATPase.⁷⁵ Solubilized ATPase is inhibited by the second group while neither oligomycin or DCCI cause any effect.⁷⁵ A third group of ATPase inhibitors are the compounds, atractyloside and bongkreikic acid. Both are specific inhibitors of ADP-ATP exchange across the membrane. Atractyloside inhibits the exchange in whole mitochondria only⁷⁶ and bongkreikic acid inhibits the exchange in submitochondrial particles only.⁷⁷

The effect of an ATPase inhibitor can be observed in a number of different ways. Other than those already mentioned, an ATPase inhibitor will have no effect on the P/O ratio.⁷⁸

Later tetradymol will be discussed as a possible ionophore.

Therefore, the last group of compounds that affect oxidative phosphorylation, ionophores, will be discussed.

There are two groups of ionophores, neutral and carboxylic. Examples of neutral ionophores are valinomycin, macrolide actins, enniatin B and 18-crown-6.⁷⁹ Nigericin, monensin and X-206 are examples of carboxylic ionophores.⁸⁰ The effects of ionophores are the same as uncouplers on mitochondria if either K^+ , Rb^+ , or Cs^+ is provided. Ionophores will cause energy dependent accumulation of those ions. Only in the presence of one of these cations will an ionophore stimulate state 4 respiration, decrease RC and P/O ratios, stimulate ATPase activity, inhibit reverse electron flow and reverse respiration inhibited by an ATPase inhibitor. The distinguishing feature between an uncoupler and an ionophore is that the activity of an ionophore is dependent upon the concentration of a cation. The activity of a true uncoupler is independent of cation concentration. The figure below illustrates this ion dependence for $2.7 \times 10^{-8} M$ valinomycin.⁷⁹

The following discussion will describe research done to determine the mode of action of tetradymol. Its uncoupling activity in whole mitochondria and its paradoxical behavior in submitochondrial particles will be described. Information from this introduction will be drawn upon in the subsequent discussion and it is hoped that the reader will appreciate some

of the lack of agreement in this field of research.

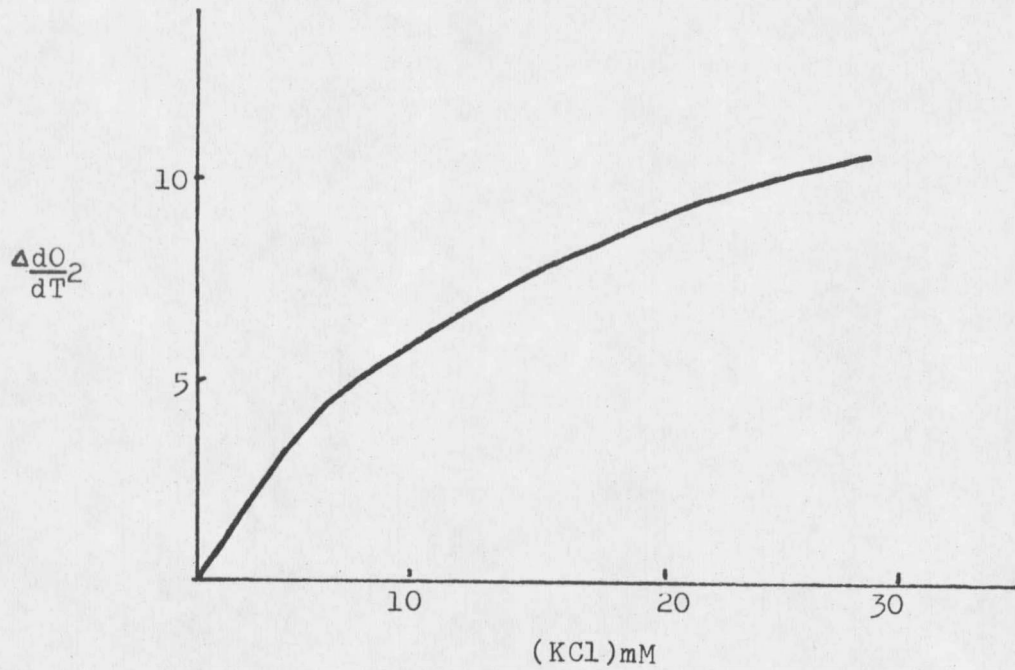


Figure 11: Dependence on K^+ concentration vs valinomycin on respiration⁷⁹

RESULTS AND DISCUSSION

As was mentioned earlier in the Introduction, the liver was implicated by histology to be the target organ in tetradymol poisoning. To further substantiate this and to gain possible information about the mode of action of tetradymol, a number of clinical tests were undertaken. In Table 5 are shown some selected results of a battery of 24 tests (the complete list is in appendix A).

Table 5

Clinical Chemistry

	<u>Control Mean</u>	<u>Drugged Mean</u>	<u>% change</u>	<u>T test P values</u>
Glucose mg%	111.7±44.50	65.0±12.10	-41.8	.1500
Indirect Bilirubin mg%	.22±0.87	.78±0.25	+225.0	.0200
Alkaline Phosphatase units	34.5±16.60	60.7±33.5	+76	.2900
Serum Glutamic Pyruvate Transaminase units	130.0±13.0	>449	>+245	.0005
Lactate Dehydrogenase	1024.5	>1147	>+12	

The elevation of indirect bilirubin (unconjugated) could be due to liver dysfunction in the conjugation process. The elevation in the serum of the three enzymes is also indicative of liver damage causing the leakage of these enzymes into the blood stream. The clinical results can be correlated to the

histological results (pg 3). SGPT is elevated more than alkaline phosphatase with no change in the thymol turbidity (appendix A). These clinical results agree with a zonal necrosis. Since the bilirubin is also up, the toxic agent(s) may be producing a mixture of cytotoxic and cholestatic damage. These results along with the previous data from Reeder,⁸¹ who demonstrated increased bromsulphalein retention times in sheep (also demonstrating damage to liver conjugation mechanisms) gave further credence to the liver being the target organ.

Since the blood glucose levels were decreased, indicating a loss of one of the sources of fuel, other aspects of energy metabolism were investigated. Holian⁸² demonstrated that liver glycogen levels, another source of fuel, were depleted. Total blood lipid levels (appendix A) are not affected. This could be due to either no net oxidation of lipids or that lipid mobilization from the fat cells is equal to lipid removal from the blood stream resulting in no net change.

Administration of glucose was considered as a possible remedy against death. The control tetradymol mean death time was 8.84 ± 1.21 hours; whereas, glucose administration resulted in a mean death time of 11.48 ± 2.22 hours ($p=0.046$). Since glucose administration "significantly protected" the mice this provided further evidence that 1) poisoned mice are suffering from an "energy shortage" and 2) tetradymol may be acting at

some aspect of energy metabolism.

Reeder⁸¹ earlier observed that sheep poisoned with tetradymol showed increased blood ammonia levels. Blood ammonia levels in control and drugged mice were measured which gave mean values of $3.3 \pm 0.6 \mu\text{g/ml}$ and $11.6 \pm 1.7 \mu\text{g/ml}$ ($p = .0024$), respectively. This 252% increase in blood ammonia was considered as a possible cause of contributing cause of death. To investigate these possibilities two approaches were used: 1) Eaton⁸³ administered NH_4^+ salts intraperitoneally to mice over a period of 7.5 hours and measured blood ammonia levels from 8 to 35 minutes after the last injection. The ammonia levels in those mice ranged from 13-50 $\mu\text{g/ml}$. Since those mice did not die, toxic levels of ammonia would be higher than those observed with the drugged mice. 2) Two whole urea cycle sparking methods were tried. Administration of N-carbamyl-L-glutamate with arginine²² resulted in a mean death time of 10.86 ± 2.51 hours ($p = .1416$), two hours longer when compared to the control death time. Arginine administration resulted in a mean death time of 10.57 ± 2.81 hours ($p = .248$). While these results did not give a highly significant confidence level, they imply a slight protection did occur. Therefore, even though the ammonia levels were not high enough to cause death, slight protection was afforded the poisoned mice by the two urea cycle sparking methods. The increased ammonia levels could be contributing

to the overall cause of death.

The possible cause for the elevation of blood ammonia was investigated. For this the effect of tetradymol on the urea cycle was looked at 'in vitro'. In Table 6 it can be seen that citrulline production from carbamyl phosphate and ornithine was decreased. The conversion of citrulline to urea though, was not affected. The conversion of citrulline to urea control mean rate was 549.3 ± 167.0 μ mole/gm protein/hour while in the presence of 2mM tetradymol the mean rate was 620.0 ± 201.1 μ mole/gm protein/hour ($p = .6114$). Reduced citrulline production could be responsible for a decreased urea cycle activity. It could be possible that the same effect might occur 'in vivo' from tetradymol toxicity which results in increased blood ammonia levels.

Since preceeding evidence suggests a defect in energy metabolism, the effects of tetradymol on mitochondrial oxidative phosphorylation and electron transport were examined to inspect the core of energy production. The remainder of the discussion is focused on these experiments. The early experiments involved possible effects on respiration. In Table 7 and figure 12 the results of varying the tetradymol concentration on state 4 respiration can be seen. The curve increases linearly at first, levels off, and starts dropping. This behavior is typical, as described earlier, for uncouplers. Maximal uncoupling ability

Table 6

Citrulline Production

<u>Condition</u>	<u>$\mu\text{mol Cit/}$ <u>mg P/ hr</u></u>	<u>T test</u> <u>P values</u>	<u>%</u> <u>change</u>
Control	3.68 $\pm$.125		
1mM Tetradymol	3.41 $\pm$.455	.1180	-14.7
2mM Tetradymol	2.53 $\pm$.187	.0019	-31.3
100 μM 2,4-DNP	2.77 $\pm$.064	.0012	-24.7

as determined from figure 12 was 350 μM and half maximal uncoupling occurred at $\sim$ 100 μM . Tetradymol, then follows the first characteristic of uncouplers by increasing state 4 respiration.

By virtue of increasing state 4 respiration, tetradymol should also reduce respiratory control (RC). The results of such experiments are given in Table 8 and figure 13. The loss of RC was rapid and complete loss occurred at 250 μM tetradymol.

The loss of P/O ratios was demonstrated in two ways. Oxygraph experiments showed complete loss of phosphorylating ability. The loss of P/O was slower than the loss of RC due to the lesser susceptibility of P/O ratios.⁸⁴ Total loss of P/O occurred approximately at maximal uncoupling ability (see Table 9 and figure 13). In the second method the ATP formed from phosphorylation was enzymatically assayed. Again, (see Table 10), it can be shown that tetradymol reduced the P/O ratios in mitochondria. Higher concentrations were necessary

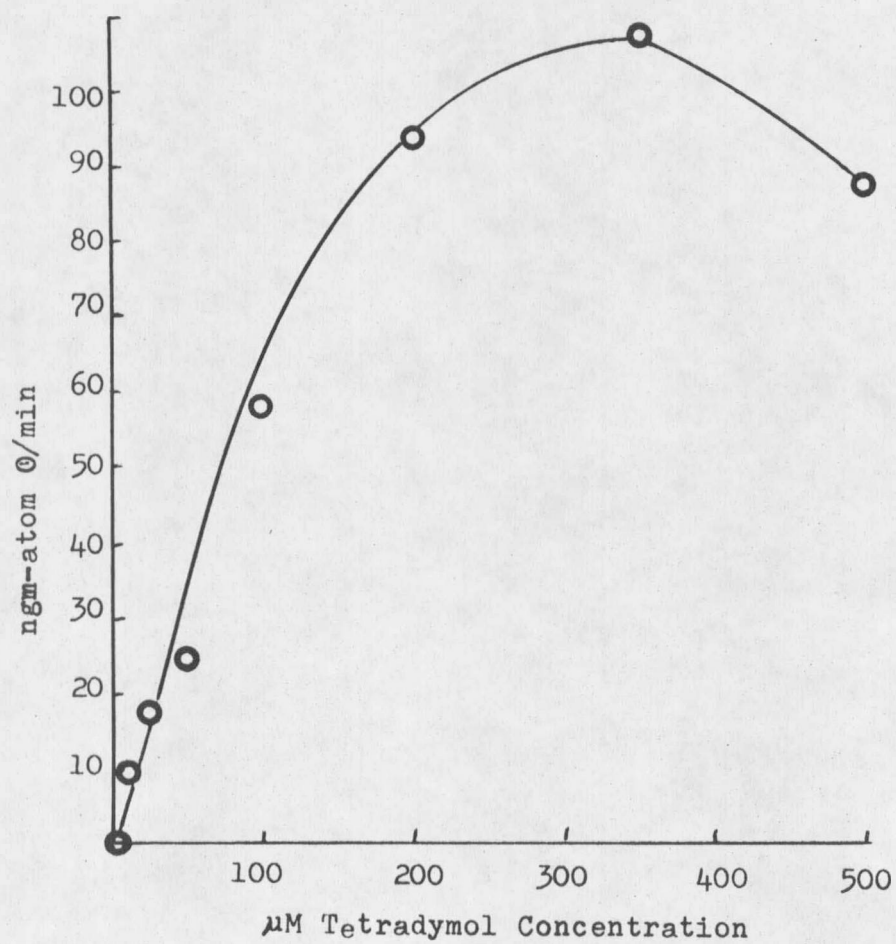


Figure 12: Effect of tetradymol on state 4 respiration

Table 7.

Effect of Tetradymol on State 4 Respiration

<u>Condition</u>	<u>ngm-atom O/min State 4</u>
1 μ M Tetradymol	0
5 μ M Tetradymol	0
10 μ M Tetradymol	9.80 $\pm$ 4.5
25 μ M Tetradymol	17.70 $\pm$ 7.8
50 μ M Tetradymol	24.58 $\pm$ 3.4
100 μ M Tetradymol	58.00 $\pm$ 13.3
200 μ M Tetradymol	94.40 $\pm$ 33.5
350 μ M Tetradymol	108.00 $\pm$ 10.0
500 μ M Tetradymol	88.10 $\pm$ 10.9

Succinate was used as substrate

due to the method of assay, i.e. any ATP formed was trapped as glucose-6-phosphate using glucose and hexokinase which shifted the equilibrium toward phosphorylation. Although complete loss was not demonstrated a significant drop was observed. In addition, high concentrations of dicoumarol and 2,4-DNP did not cause complete loss of P/O ratios. Therefore, both methods established that tetradymol reduced the P/O ratio and, consequently, fulfilled the second characteristic of uncouplers.

Since most of the classical uncouplers act nearly equally at the three sites of phosphorylation, it was of interest to

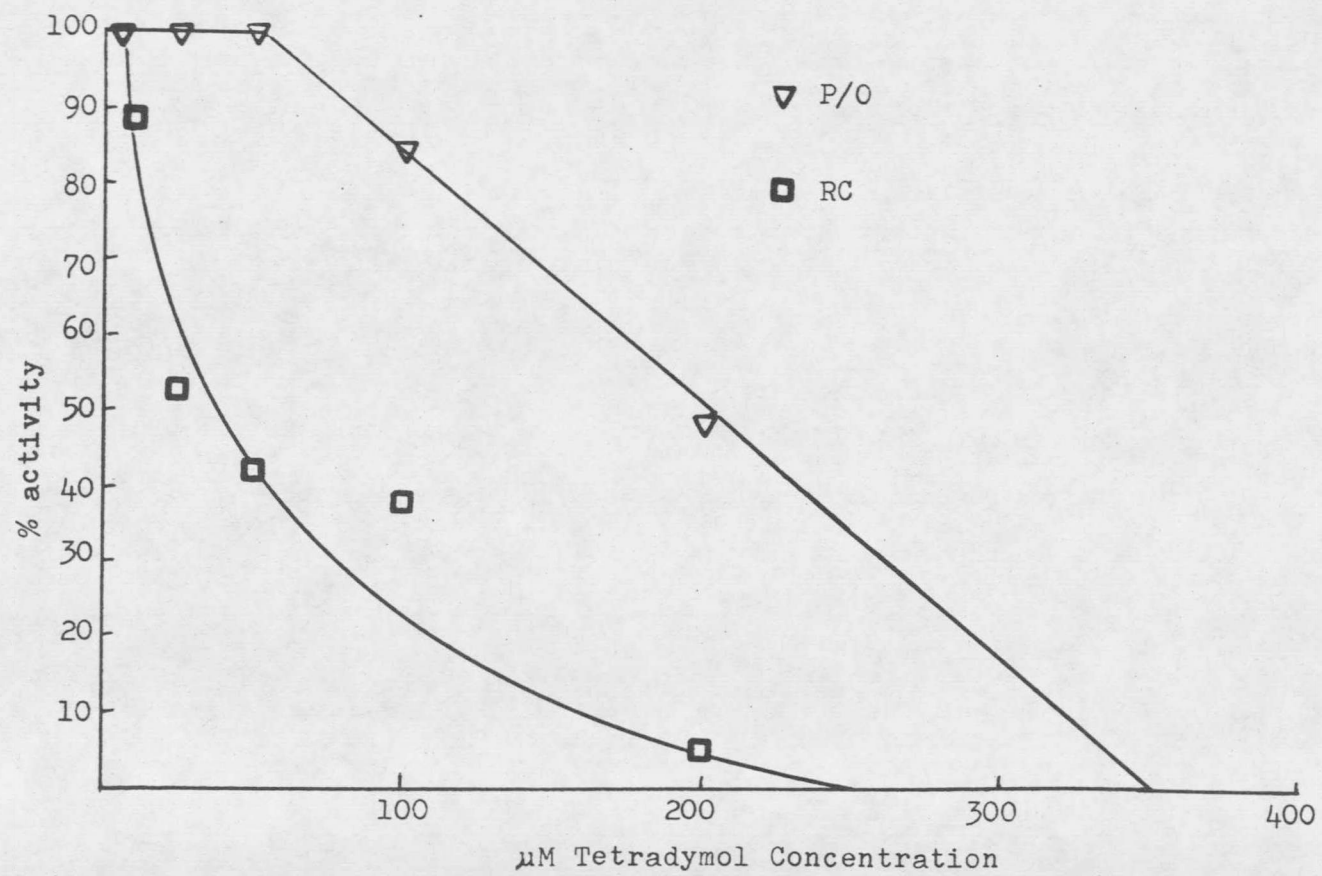


Figure 13: Effect of tetradymol on RC and P/O

Table 8

Effect of Tetradymol on RC

<u>Condition</u>	<u>RC Mean</u>	<u>T test P values</u>	<u>% change</u>
Control	4.53±.450		
1μM Tetradymol	4.56±.550	.937	0
5μM Tetradymol	4.50±.099	.902	0
10μM Tetradymol	4.43±.635	.828	0
25μM Tetradymol	3.53±.150	.023	-28
50μM Tetradymol	3.23±.550	.034	-37
100μM Tetradymol	2.17±.193	.002	-67
200μM Tetradymol	1.26±.138	.001	-93
350μM Tetradymol	1.00±0	.001	-100
500μM Tetradymol	1.00±0	.001	-100

Succinate was used as substrate

check the action of tetradymol at the three sites. In Table 11 the results of these experiments are given. At all three sites, 200μM tetradymol almost completely reduced the RC and partially reduced the P/O ratio. At 350μM both the RC and P/O ratio were completely lost. At 200μM the loss in the P/O ratio at each of the three sites was determined. The loss in the P/O ratio was 23%, 21%, and 27% for the first, second, and third sites, respectively. Since the activity at each site was approximately the same, the mode of action of tetradymol at

Table 9

Effect of Tetradymol on P/O Ratios

<u>Condition</u>	<u>P/O Mean</u>	<u>T test P values</u>	<u>% change</u>
Control	1.92±.046		
1μM Tetradymol	1.91±.028	.9245	0
5μM Tetradymol	1.87±.071	.3951	0
10μM Tetradymol	1.91±.038	.9245	0
25μM Tetradymol	1.90±.060	.6705	0
50μM Tetradymol	1.91±.065	.8322	0
100μM Tetradymol	1.62±.075	.0055	-15.6
200μM Tetradymol	0.84±.040	.0003	-56.3
350μM Tetradymol	0	.0003	-100.0
500μM Tetradymol	0	.0003	-100.0

Succinate was used as substrate

the three sites must be approximately the same. This indicates that both the first and second characteristics of uncouplers are observed at all three sites of phosphorylation.

The third characteristic of uncouplers is the ability to restore increased oxygen uptake rates of inhibited ATPase. Tetradymol reversed the inhibition by both oligomycin and DCCI (Table 12). In both cases addition of varying concentrations of tetradymol caused significant increases in rates of oxygen uptake. From these observations one can conclude tetradymol

Table 10

Effect of Tetradymol on P/O Ratios

<u>Condition</u>	<u>P/O Mean</u>	<u>T test P values</u>
Control	1.335±0.50	
0.5mM Tetradymol	2.015±.021	.0022
1.0mM Tetradymol	0.935±.120	.0460
1.5mM Tetradymol	0.865±0.21	.0046
2.0mM Tetradymol	0.720±0.57	.0052
10µM Dicoumarol	0.820±.028	.0042
20µM Dicoumarol	0.470±.042	.0020
50µM 2,4-DNP	0.905±.035	.0072

Succinate was used as substrate

has uncoupled the electron transport chain from the inhibited oxidative phosphorylation process and allowed increased oxygen uptake rates. Tetradymol, then fulfilled the third characteristic of uncouplers.

The fourth characteristic contains two parts for both mitochondria and submitochondrial particles. Discussion of submitochondrial particles is given later.

In mitochondria it should be demonstrated that ATPase can be stimulated and that reverse electron flow can be inhibited, if tetradymol behaves as a true uncoupling agent. The stimulation of ATPase activity as a function of tetradymol con-

Table 11

<u>Relative Uncoupling at 3 Sites</u>				
<u>Condition</u>	<u>RC Mean</u>	<u>P/O Mean</u>	<u>T test</u> <u>P values</u>	<u>% Loss</u>
<u>Glutamate</u>				
Control	3.76 ±.26	2.74 ±.38		
200uM Tetradymol	1.39 ±.21	2.11 ±.12	.0238	23
350uM Tetradymol	1.0 ±0	0	.0	100
<u>Succinate</u>				
Control	5.37 ±.46	1.82 ±.06		
200uM Tetradymol	2.07 ±.32	1.43 ±.06	0	21
350uM Tetradymol	1.0 ±0	0	0	100
<u>Ascorbate/TMPD</u>				
Control	1.37 ±.10	0.68 ±.07		
200uM Tetradymol	1.13 ±.06	0.49 ±.08	.0064	27
350uM Tetradymol	1.0 ±0	0	0	100

Table 12a

Reversal by Tetradymol of DCCI Inhibited Mitochondrial ATPase

<u>Condition</u>	Mean <u>ngm-atom O/min</u>	T test <u>P values</u>
Control	78.87±2.47	
200uM Tetradymol	122.6±6.0	.0011
Control	77.8±3.15	
10uM Dicoumarol	274.8±12.7	.0016

Table 12b

Reversal by Tetradymol of Oligomycin Inhibited Mitochondrial ATPase

<u>Condition</u>	Mean <u>ngm-atom O/min</u>	T test <u>P values</u>
Control	81.2±9.38	
200uM Tetradymol	117.4±20.30	.0491
400uM Tetradymol	184.9±10.60	.0009
Succinate was used as substrate		

centration is shown in Table 13. The maximum stimulation provided by tetradymol was greater than that of 2,4-DNP, a common uncoupler.

In Table 14 the effect of tetradymol on reverse electron flow can be seen. Much lower concentrations were sufficient to completely inhibit this reaction. The ATPase inhibitor, oligomycin, also caused complete reduction of activity. Therefore, since tetradymol causes both increased ATPase activity and decreased reverse electron flow, the fourth characteristic of

Table 13

Mitochondrial ATPase Activity

<u>Condition</u>	<u>ATPase Activity Mean mg. Pi/mg Protein/hr</u>	<u>T test P values</u>	<u>% change</u>
Control	1.26±.010		
.5mM Tetradymol	1.63±.059	.0101	+29.1
1mM Tetradymol	2.42±.071	.0014	+91.1
2mM Tetradymol	4.92±.040	.0002	+289.0
100uM 2,4-DNP	4.61±.030	.0002	+265.0
200uM 2,4-DNP	4.19±.129	.0002	+232.0

Table 14

Reverse Electron Flow in Mitochondria

<u>Condition</u>	<u>NADH Fluorescence Arbitrary vertical units - Mean</u>	<u>T test P values</u>
Control	44.6±5.2	
33uM Tetradymol	0	.0007
133uM Tetradymol	0	.0007
10uM 2,4-DNP	0	.0007
123uM Oligomycin	0	.0007

Succinate was used as substrate.

uncouplers in mitochondria was fulfilled.

The fifth and last characteristic of uncouplers applies to the physical properties of the uncoupler itself, i.e. weak acids (pKa 3-9) and usually lipid soluble. Tetradymol is organic

soluble (hexane, ether, ethanol, methanol and etc.) and practically insoluble in water. Therefore, it does fit the category of lipid solubility.

Tetradymol should have a relatively high pKa. The most acidic proton should be on the tertiary hydroxyl group. Among some known compounds, t-butyl alcohol has a pKa of 19, ethanol has a pKa of 18, and methanol has a pKa of 16.⁸⁵ The next most acidic proton on tetradymol should be an allylic proton which should have a pKa of 21 or higher.⁸⁵ The relative acidity of tetradymol was compared to 4-chloro-2-nitroaniline having a pKa of 17.2.⁸⁶ These experiments resulted in Keqs favoring the 4-chloro-2-nitroaniline anion, $K_{eq} = 172$ ($\log = 2.24$). for the 4-chloro-2-nitroaniline anion with tetradymol reaction, $K_{eq} = 94$ ($\log = 1.97$), for the 4-chloro-2-nitroaniline with tetradymol anion reaction. The reliability of these types of experiments is no greater than pKa differences of two.⁸⁷ Therefore, the pKa of tetradymol is greater than 17.2 and probably close to the pKa of 19 predicted for the proton of a tertiary hydroxyl group. Tetradymol, then, has a pKa well outside of the 3-9 range found for classical uncouplers. This is the one distinction that tetradymol is unique as an uncoupler by having a different physical property.

It is important to point out that previously noted observations indicate that tetradymol has uncoupler like activity

in mitochondria. Earlier in the discussion of urea cycle activity it was established that conversion of ornithine and carbamyl phosphate to citrulline was reduced. There are two possible explanations. Actual inhibition of ornithine trans-carbamylase or alternatively inhibition of ornithine transport. The latter has been noted for the uncoupler, 2,4-DNP.²⁹ The transport process is linked to the electron transport chain. With the evidence presented here no definite conclusion can be drawn as to which of the two alternative explanations is more correct, but it could be the uncoupling property of tetradymol which caused the ultimate reduction of citrulline production.

It is important to establish, having a relatively weak uncoupler (compared to classical uncouplers like dicoumarol, carbonyl cyanides and etc.), that the uncoupling ability that is present is not due to a detergent effect on the mitochondrial membrane. A detergent would solubilize the membrane causing a change in the optical properties of a suspension of mitochondria. The effect of 2,4-DNP, SDS, tetradymol in ethanol, and an equivalent amount of ethanol on the optical density of a dilute suspension of mitochondria can be seen in Table 15. SDS was the only compound of the four that caused a significant change in the optical properties of the mitochondria. What change tetradymol did have was attributed to ethanol. A concentration of 2,4-DNP, sufficient to cause

Table 15

Effect of Tetradymol on Mitochondria

<u>Condition</u>	<u>Mitochondria (OD)</u>	<u>Mitochondria & Affector (OD)</u>	<u>T test P values</u>
25uM 2,4-DNP	0.98±.046	0.932±0.25	.2002
100uM SDS	0.997±0.47	0.867±.038	.0214
100uM Tetradymol	0.987±.050	0.935±0.48	.2685
.2% Ethanol	0.977±.029	0.938±.019	.1263

near maximal uncoupling, exhibited a slight effect. At 100uM SDS (Table 16) no uncoupling ability could be demonstrated; whereas, 150uM tetradymol had approximately the same uncoupling ability as 400uM SDS. Considering these observations, it is not likely that tetradymol's uncoupling effect is due to a detergent-like action on the mitochondria.

Table 16

Comparison of SDS and Tetradymol Uncoupling

<u>Condition</u>	<u>State 4 ngm-atom O/min</u>	<u>Uncoupler ngm-atom O/min</u>	<u>T test P values</u>
100uM SDS	65.45±2.05	65.45±2.05	1.0
100uM Tetradymol	64.01±4.25	99.23±3.25	.0043
400uM SDS	62.50±2.12	113.5±4.10	.0028
150uM Tetradymol	56.2±6.93	114.95±2.05	.0053

Succinate was used as substrate

Many ionophores exhibit uncoupling activity (pg 27); however, this activity is enhanced by increasing the K^+ concentration. If tetradymol had any ionophoric properties then a similar behavior in the uncoupling ability would be observed as a function of K^+ concentration. In Table 17 it can be seen that varying the K^+ concentration from 0-20mM had no significant effect on activity. Since there was no change it can be concluded that tetradymol was not functioning as an ionophore.

Table 17

Varying K^+ Concentration

<u>Condition</u>	<u>Uncoupled rate</u>	
	<u>State 4</u>	<u>T test</u>
	<u>ngm-atom O/min</u>	<u>P values</u>
0mM K^+	41.6±7.83	
5mM K^+	34.1±6.88	.2425
10mM K^+	36.0±8.0	.3589
15mM K^+	29.9±1.85	.0547
20mM K^+	39.5±6.6	.7192

Succinate was used as substrate

Since the pKa of tetradymol is considerably removed from the range of 3-9 there should not be any significant change in the uncoupling ability in varying the pH of the medium. In Table 18 and figure 14 the effect of varying the pH of the medium on state 3, state 4, and the uncoupling ability of 200μM

tetradymol can be seen. State 4 remained relatively constant while both state 3 and RC were optimal around pH 7.0-7.5. The uncoupling ability of tetradymol followed the same pattern with its maximal activity at pH 7.0 and drops off on both sides. The change in uncoupling ability that did occur has been attributed by Wilson⁸⁸ as intrinsic mitochondrial uncoupling ability. Tetradymol does not show any change in uncoupling ability with pH as would be predicted for a classical uncoupler.

Table 18

Tetradymol Uncoupling vs pH

pH	Control State 3 ngm-atom O/min	Control State 4 ngm-atom O/min	Uncoupled State 4 ngm-atom O/min	Control RC
6.0	273.5±5.65	82.7±1.62	165.5±1.63	3.31
6.5	345.9±11.37	78.0±1.67	203.1±4.91	4.43
7.0	397.6±9.81	81.8±2.80	227.5±25.45	4.86
7.5	418.3±4.3	89.3±1.62	202.1±21.29	4.68
8.0	345.0±15.52	79.0±0	195.5±25.45	4.37
8.5	284.0±10.19	77.1±1.07	193.6±26.23	3.69

Succinate was used as substrate

To obtain the maximal binding ratio of tetradymol to mitochondria, the uncoupling ability was observed as the mitochondrial protein was varied (Table 19 and figure 15).

At low mitochondrial protein concentrations the graph was

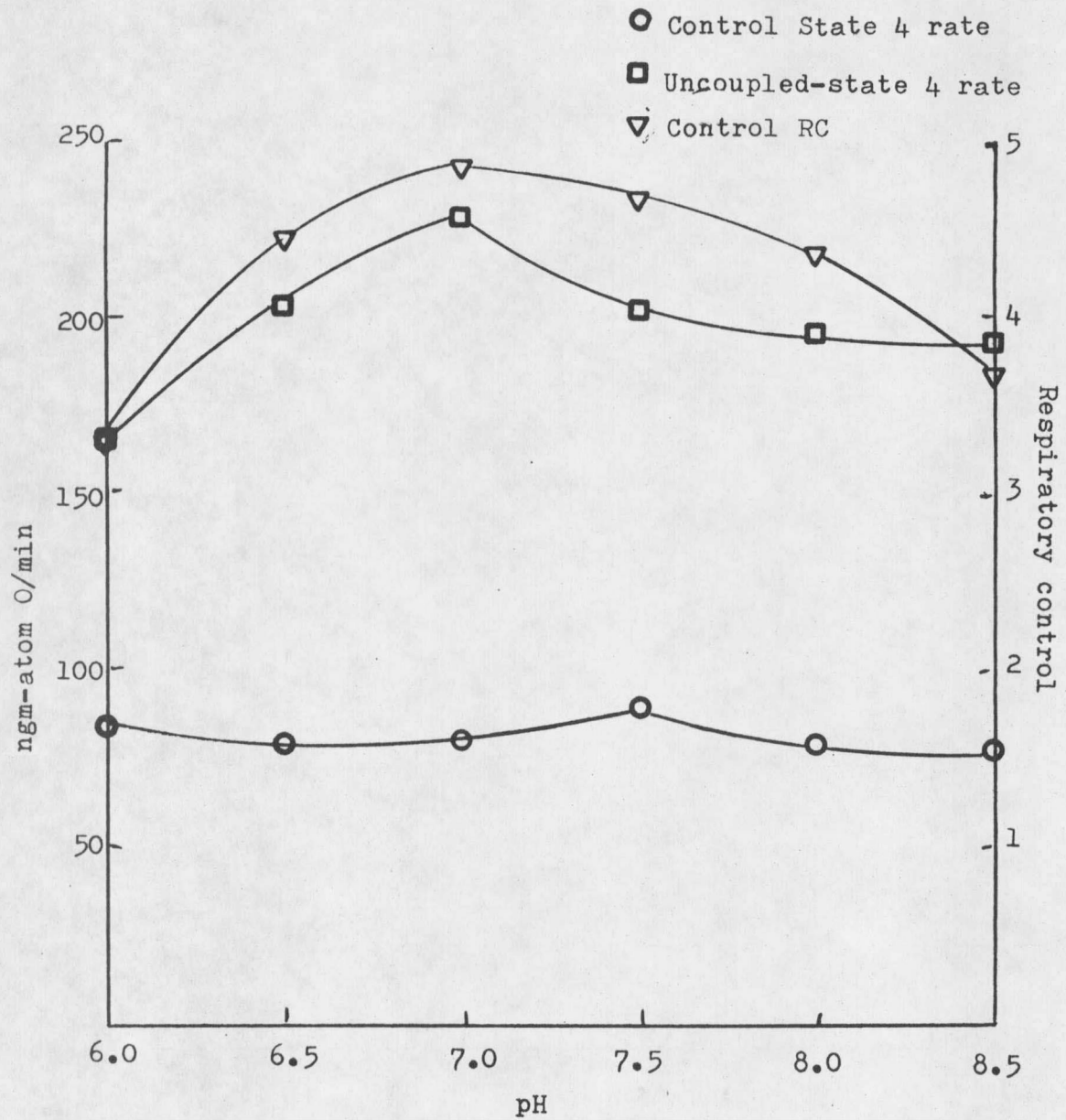


Figure 14: Tetradymol uncoupling vs pH

Table 19

Tetradymol Uncoupling vs Protein

<u>mg P</u>	<u>State 4</u> <u>ngm-atom O/min</u>	<u>Uncoupled</u> <u>ngm-atom O/min</u>	<u>Difference</u> <u>ngm-atom O/min</u>
1.07	13.0±3.46	50.0±4.58	37.0±6.25
1.82	43.0±1.73	100.0±3.46	57.0±3.0
2.14	44.0±6.25	110.0±10.35	66.0±5.20
2.68	61.5±6.36	142.5±6.36	81.0±0
3.21	79.0±3.46	172.0±3.46	93.0±0
4.28	110.0±1.73	220.0±3.46	110.0±4.58
5.35	139.0±9.16	249.9±9.0	110.0±16.52
6.42	177.0±7.94	294.0±7.94	117.0±7.94

Succinate was used as substrate

linear then leveled off at higher concentrations. This leveling off indicated that the amount of binding sites was saturating with respect to tetradymol. The point at which it started to level off, then, would be the optimal ratio of tetradymol binding for uncoupler activity. This value was 0.133 μ mole/mg protein.

BSA has the property of adsorbing uncouplers and returning uncoupled mitochondria to their normal state 4 respiration. In Table 20a and b, the effect of BSA on either 2,4-DNP or tetradymol uncoupled mitochondria and the effect of pretreating with BSA can be seen. BSA added after 2,4-DNP returned the mitochondria back to the normal state 4 rates. On the other hand,

Table 20a

Effect of BSA

<u>Condition</u>	B rate after Uncoupler <u>ngm-atom O/min</u>	C rate after 1% BSA <u>ngm-atom O/min</u>	B vs C T test <u>P values</u>
200uM 2,4-DNP	410.3±8.20	69.85±4.2	.0004
200uM Tetradymol	119.3±4.10	139.7±4.10	.0348
150uM Tetradymol	109.6±9.38	129.0±8.43	.0559
400uM Tetradymol	254.6±14.4	359.4±2.06	.0070

Succinate was used as substrate

Table 20b

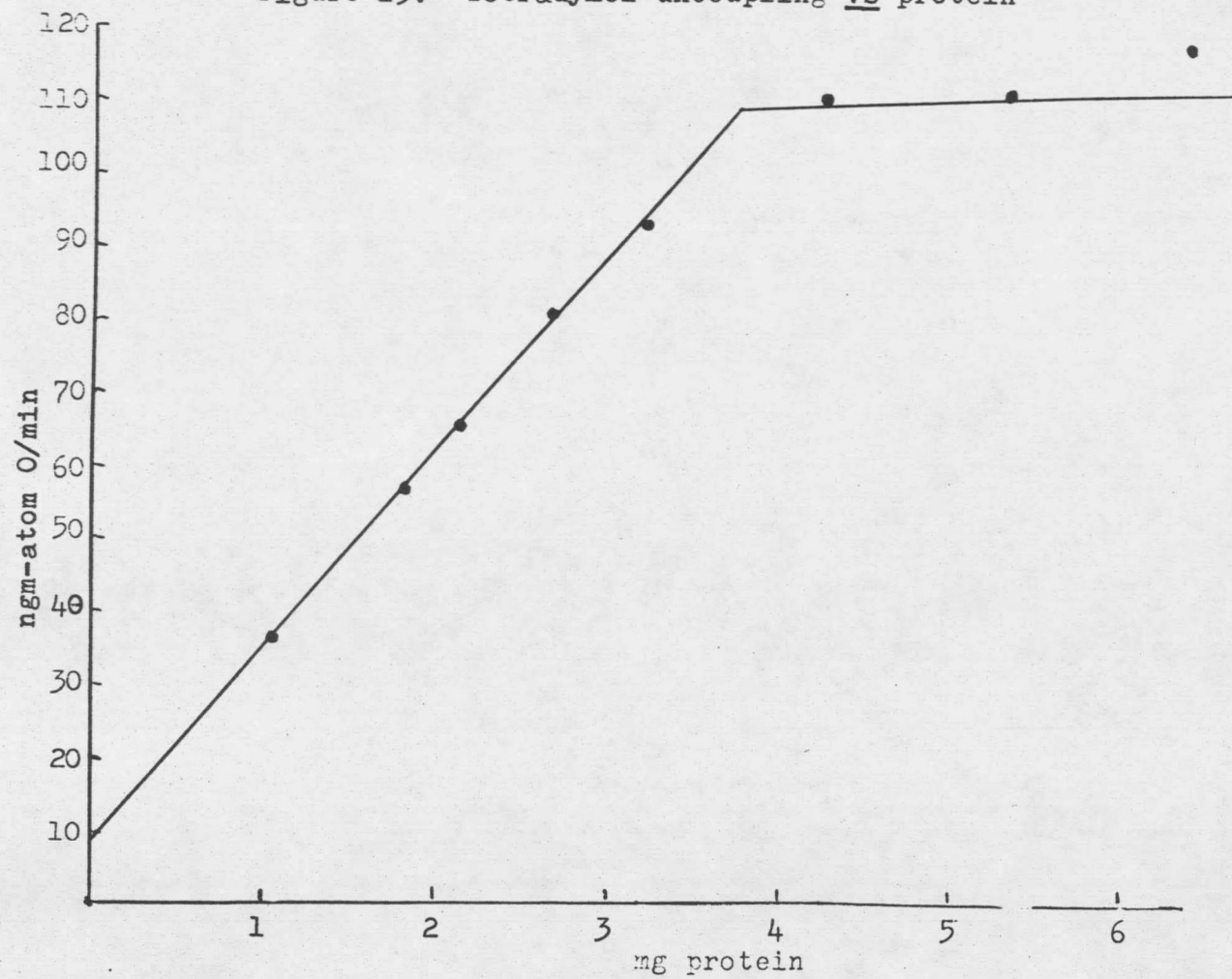
Effect of BSA

<u>Condition</u>	D Uncoupled rate <u>ngm-atom O/min</u>	B vs D T test <u>P values</u>
200uM Tetradymol	75.65	.0662
400uM Tetradymol	139.7	.0076

Succinate was used as substrate

BSA added after tetradymol actually increased the oxygen uptake rates significantly. If BSA was added before tetradymol there was a significant protection afforded against uncoupling but not complete protection. These latter results could indicate that tetradymol could not readily bind to BSA or that it has a greater affinity for mitochondria than BSA. The results from post addition of BSA suggested that the latter hypothesis was

Figure 15: Tetradymol uncoupling vs protein



correct since there was no reversal. Overall, the binding of tetradymol to mitochondria appeared to be very tight. In fact, when BSA was added after tetradymol the rate increased, this suggested BSA was removing something that allowed tetradymol to be more efficient as an uncoupler. Possibly BSA may remove lipid material from the mitochondria allowing increased binding of tetradymol. However, these observations are not well understood at this time.

It would be expected that an uncoupler would stimulate the tricarboxylic acid cycle since it would reduce the NADH/NAD^+ ratio. Experiments to demonstrate a stimulated TCA cycle with tetradymol and known uncouplers following the conversion of pyruvate labeled on the first or second carbon to $^{14}\text{CO}_2$ were unsuccessful. Various workers^{89,90,91,92} using labeled substrates have shown conflicting results on TCA cycle stimulation by uncouplers.

The fourth characteristic of uncouplers is that ATPase activity in both mitochondria and submitochondrial particles is stimulated. Also, reverse electron flow in both particles and mitochondria should be inhibited. Tetradymol does inhibit reverse electron flow (Table 21) to at least 85% in submitochondrial particles. Increasing the concentration of tetradymol increased the inhibition. Dicoumarol and 2,4-DNP also inhibited reverse electron flow. The ATPase inhibitors oligomycin and

Table 21

<u>Reverse Electron Flow in Submitochondrial Particles</u>			
<u>Condition</u>	<u>Rates nM NADH/min</u>	<u>% change</u>	<u>T test P values</u>
Control	87.1 $\pm$ 3.15		
33 μ M Tetradymol	63.4 $\pm$ 2.19	-27.3	.0023
66 μ M Tetradymol	37.2 $\pm$ 1.13	-57.3	.0003
133 μ M Tetradymol	31.7 $\pm$ 2.26	-63.6	.0003
266 μ M Tetradymol	12.7 $\pm$ 2.26	-85.4	.0002
33 μ M 2,4-DNP	20.3 $\pm$ 3.39	-66.4	.0003
66 μ M 2,4-DNP	18.2 $\pm$ 3.39	-79.1	.0002
133 μ M 2,4-DNP	1.6 $\pm$ 2.26	-98.2	.0001
33 μ M Dicoumarol	24.55 $\pm$ 1.06	-71.8	.0002
123 μ M Oligomycin	3.2 $\pm$ 0	-96.3	.0001
98 μ M DCCI	19.8 $\pm$ 10.0	-77.3	.0011

DCCI also inhibited reverse electron flow. Therefore, tetradymol, by inhibiting reverse electron flow in submitochondrial particles, followed that part of the fourth characteristic.

The last part of the fourth characteristic gave conflicting results (Table 22). Instead of the expected increase of ATPase activity, as with 2,4-DNP, tetradymol inhibited ATPase activity. Tetradymol also inhibited 2,4-DNP stimulated ATPase activity. Thus tetradymol did not parallel expected uncoupler activity in this instance.

Table 22

Submitochondrial ATPase Activity

<u>Condition</u>	<u>mg Pi liberated/ mg protein/hr</u>	<u>% change</u>	<u>T test P values</u>
Control	4.04±.611		
1mM Tetradymol	0.46±.311	-89	.0002
200µM 2,4-DNP	6.15±.416	+46	.0024
1mM Tetradymol + 200µM 2,4-DNP	1.00±.78	-76	.0062

Further support for ATPase inhibition in submitochondrial particles came from having looked at oxygen uptake rates (see Table 23). By increasing the tetradymol concentration the oxygen uptake by submitochondrial particles was decreased, not increased. The inhibition provided by 250µM tetradymol was completely reversed by 10µM dicoumarol. Therefore, this activity of tetradymol was also like that of an ATPase inhibitor.

The second characteristic of uncouplers which states that respiratory chain linked phosphorylation is inhibited should be followed in submitochondrial particles. The effects of tetradymol and known effectors on ATPase activity are listed in Table 24. A loss of P/O ratio could be shown with 2,4-DNP and dicoumarol as expected. Tetradymol, though, showed no loss of P/O ratio. Oligomycin also showed no significant loss of P/O ratio. This indicated that in this experiment tetradymol again was not acting as an uncoupler but rather as an ATPase inhibitor. This could

Table 23

Effect of tetradymol on Submitochondrial
Oxygen Consumption Rates

<u>Condition</u>	<u>A</u> [*]	<u>B</u> ^o	<u>P</u> <u>values</u>	<u>P/O</u> <u>dec</u>	<u>C</u> ⁺	<u>P</u> <u>values</u>	<u>%</u> <u>rev</u>
250uM Tetradymol	251.3 ±17.2	200.7 ±14.7	.0179	60	253.0 ±1.7	.0040	100
500uM Tetradymol	257.3 ±8.3	136.3 ±4.3	.0009	47	149.0 ±5.2	.2211	11
750uM Tetradymol	254.3 ±18.5	66.0 ±4.6	.0006	74	110.7 ±5.1	.0011	24

Succinate was used as substrate

* ngm-atom O/min State 4

^o ngm-atom O/min after tetradymol

⁺ ngm-atom O/min 10uM dicoumarol

be the same interpretation of the results of reverse electron flow, since ATPase inhibitors could produce the same results.

In appendix B the structures of a number of related compounds are shown. These were investigated with respect to a number of mitochondrial activities to see how they acted and if any polar or stereochemical agreement concerning activity could be made. It can be seen in Table 21 that tetradymadiol monoisobutyrate had no effect on P/O in submitochondrial particles. While this did not suggest any activity it did show that up to 2mM it did not exhibit this kind of uncoupling ability in submitochondrial particles.

Table 24

P/O in Submitochondrial Particles

<u>Condition</u>	<u>P/O values</u>	<u>T test</u> <u>P values</u>
Control	.51±.063	
1mM Tetradymol	.615±0.50	.1115
2mM Tetradymol	.538±.054	.5370
1mM Tetradymadiol Monoisobutyrate	.557±.32	.2990
2mM Tetradymadiol Monoisobutyrate	.545±.64	.5590
186μM Oligomycin	.465±.007	.3960
100μM 2,4-DNP	.225±.007	.0051
200μM 2,4-DNP	.157±.035	.0008
20μM Dicoumarol	.220±0	.0048

When the submitochondrial ATPase activity was investigated (Table 25) tetradymadiol monoisobutyrate did exhibit ATPase inhibition but to a lesser extent than tetradymol. Menthofuran inhibited ATPase to a greater extent than the ester. Menthofuran also inhibited 2,4-DNP stimulated ATPase activity. Since the RF (appendix B) values in 70% ether-30% hexane increased in going from tetradymol to tetradymadiol monoisobutyrate to menthofuran, there may be a similar pattern in the ATPase inhibitory properties. In reality tetradymadiol monoisobutyrate has the least activity, the bulky isobutyl ester

Table 25

Submitochondrial ATPase Activity

<u>Condition</u>	<u>mg Pi liberated/ mg protein/hr</u>	<u>T test P values</u>	<u>% change</u>
Control	4.04±.611		
1mM Tetradymol	0.46±.311	.0002	-89
1mM Tetradymadiol Monoisobutyrate	2.74±.558	.0230	-35
1mM Menthofuran	1.27±.019	.0010	-70
200μM 2,4-DNP	6.15±.416	.0024	+46
1mM Menthofuran + 200μM 2,4-DNP	3.63±.035	.2748	-14

reduced some of its activity. This could indicate some stereochemical requirement of the inhibitory site rather than activity dependent on TLC mobility.

Further support for stereochemical requirement was gained by looking at mitochondrial ATPase activity (Table 26). The same pattern for activating mitochondrial ATPase can be seen. Again tetradymadiol monoisobutyrate exhibited the lowest activity and menthofuran was slightly lower than tetradymol. Cleaving the ester to give tetradymadiol I slightly increased the activity even though it has a much lower R_f value. Therefore, removing part of the group improves the stereochemistry for the binding site. Oxidizing the diol I to the ketone or conversion to the other diol II, reduced the activity consider-

Table 26

Mitochondrial ATPase Activity

<u>Condition</u>	<u>mg Pi liberated/ mg protein/hr</u>	<u>% change</u>	<u>T test P values</u>
Control	0.37±.049		
1mM Tetradymol	2.07±.340	+469	.0161
1mM Tetradymadiol Monoisobutyrate	0.90±.140	+145	.0337
1mM Tetradyma Diol I	1.11±.092	+202	.0072
1mM Tetradyma Diol II	0.68±.230	+86	.1953
1mM Tetradyma Ketone	0.59±.177	+60	.2330
1mM Oxidized Tetradymol	0.51±.707	+38	.1413
1mM Reduced Tetradymol I	1.37±.092	+275	.0038
1mM Reduced Tetradymol II	.0395±.007	+7.3	.4866
1mM Reduced Tetradymol III	0.42±.042	+15	.3560
1mM Menthofuran	1.66±.270	+349	.0192
1mM Benzofuran	0.60±.071	+62	.0592
1mM Peperic acid	0.355±.021	0	.8093
200uM 2,4-DNP	2.89±.049	+690	.0005

ably even though the R_f value had increased slightly. It became apparent that this movement of the oxygen must affect the binding ability (stereochemical specificity) to the uncoupler site. The CrO_3 oxidation product of tetradymol, having a very low R_f value, exhibited very low activity. Peperic acid, the CrO_3 oxidation product of menthofuran, had no activity. In these oxidized products part of the unsaturation of the furan ring was lost. If the furan ring was reduced as in the three reduced products, loss of ATPase stimulation was also produced. The first reduced product has an R_f just below tetradymol with only about half of the activity. The other two compounds had almost no activity. Therefore, it appeared as though saturation of the furan ring may decrease the activity. On the other hand Benzofuran, having more unsaturation, did not have a higher activity than tetradymol or menthofuran. Therefore, either saturation of the B ring may be important or benzofuran may not be a good model.

These compounds were also examined with respect to increasing state 4 respiration (Table 27). In this experiment very similar patterns were observed. Tetradymadiol monoisobutyrate activity was lower than tetradymol. Diol I was more active than the ketone and diol II. Oxidized tetradymol and peperic acid had very low activity. Benzofuran had a small amount of activity. Tetradymol reduced compounds' activities

Table 27

Relative Uncoupling Ability of Tetradymol-like Compounds

<u>Condition</u> <u>500uM each</u>	<u>Increase of rate</u> <u>over State 4</u>	<u>Rel. rate to</u> <u>Tetradymol</u>
Tetradymol	303.0±4.24	1.00
Tetradymadiol Monoisobutyrate	192.0±0	0.63
Tetradyma Diol I	36.0±4.24	0.12
Tetradyma Diol II	4.5±0	0.015
Tetradyma Ketone	15.0±0	0.05
Tetradymol Reduced I	54.0±4.24	0.18
Tetradymol Reduced II	66.0±4.24	0.22
Tetradymol Reduced III	63.0±0	0.21
Oxidized Tetradymol	28.5±6.36	0.09
Menthofuran	399.0±38.2	1.12
Benzofuran	33.0±0	0.11
Peperic Acid	15.0±0	0.05

Succinate was used as substrate

were greatly reduced but the distinction between the first and the other two was lost. This further emphasizes the loss of activity with saturation of the furan ring. The major difference was that menthofuran was stronger in increasing state 4 respiration than tetradymol. Thus menthofuran may be a more potent uncoupler than tetradymol.

Since menthofuran had high uncoupling activities, the toxicity of menthofuran was investigated. It was found that oral administration of 300mg/kg resulted in a mean death time of 13.5 ± 0.5 hours. Histologically the liver showed damage similar to tetradymol poisoning except much milder.

Lee⁹³ administered 300mg/kg of the three reduced compounds and observed that none of the mice died. Mice given 800mg/kg of tetradymadiol monoisobutyrate orally resulted in a mean death time of 10.7 ± 1.23 hours. Histologically the damage to the liver was mild centralobular necrosis.

Conclusion - The clinical findings in this report further substantiate earlier observations that ingestion of tetradymol causes a zonal (centralobular) necrosis in the liver. Additionally, mild cholestasis accompanies these necrotic changes. In intact mitochondria tetradymol followed all the characteristics of an uncoupler except having a pKa too far removed from the 3-9 range. This high pKa would account for the lack of uncoupling activity dependence as a function of pH. Also,

since menthofuran had similar uncoupling activity to tetradymol and its most acidic proton (allylic) would be expected to be a weaker acid than the hydroxyl proton on tetradymol, there is even less reason to suspect any protonophoric mechanism of uncoupling for tetradymol in which a low pKa would be necessary. Therefore, either the pKa characteristic of uncouplers should be treated loosely or tetradymol is a unique uncoupler. The uncoupling activity in mitochondria would explain the reduced glucose levels in animals ingesting tetradymol. Uncouplers inhibit ornithine transport and thus inhibit citrulline production. This would account for the increased ammonia levels. One can also argue that ammonia levels may have contributed to the death of the animal; however, this was probably not a primary cause of death. It is proposed here that the cause of death could be due to the uncoupling action of tetradymol. Uncouplers should reduce the ATP levels by preventing ATP synthesis and increasing ATP hydrolysis (ATPase activity). Lack of ATP has been shown to cause membrane disruption⁹⁴ which could produce the observed liver necrosis. Reduced ATP levels would impair all ATP requiring reactions.

In submitochondrial particles the evidence presented on the action of tetradymol is contradictory to the action of uncouplers. Tetradymol, instead, behaved as an ATPase inhibitor. Tetradymol reduced ATPase activity, reverse electron

flow, and oxygen uptake rates that could be reversed by an uncoupler and had no effect on P/O ratios, all of which are characteristic of ATPase inhibitors.

The experiments with BSA suggests that tetradymol is a very tight binder to mitochondria. Therefore, because the effects of tetradymol are opposite on the two sides of the inner membranes of the mitochondria it may bind tightly to one side or the other, exert its effect, and not cross over. This would further suggest that tetradymol's uncoupler binding site is on the outside and not the inside of the mitochondrial membrane as suggested for other uncouplers.

Together with all evidence it is suggested that tetradymol is unique and should be put into a new classification of uncouplers. It is possible that a large number of other toxic hydrocarbon natural product compounds that have not been investigated could fall into this class of uncouplers. This could become an important class of uncouplers since most of the uncouplers available are not natural products. This new class would then represent a more natural situation.

Investigations with related compounds have shown that uncoupling ability can not be correlated to TLC mobility (R_f). Therefore, conclusions were drawn concerning the stereochemical specificity of the tetradymol binding sites. 1) The unsaturated furan ring seems to be important. 2) A group on the six

position reduced the activity and further 3) putting a group down on that carbon even further reduces the activity. 4) The A ring and hydroxyl group of tetradymol are not essential since menthofuran has similar activities.

EXPERIMENTAL

I. Reagents

For the citrulline assay 2,3-butanedione-2-oxime (Eastman) was used as the color reagent. It was recrystallized three times from water before use.

For the urea assay 1,2-propanedione-1-phenyl-2-oxime was used as a color reagent. It was prepared according to the procedure in Organic Synthesis Collected Volume 2.⁹⁵

For the determination of the pKa of tetradymol, 4-chloro-2-nitroaniline (Chem Service) was recrystallized twice from 100% ethanol before use as a standard.

Tetradymol was isolated following the procedure described by Dr. Reeder.² Tetradymadiol monobisobutyrate, tetradymadiol I, -diol II and -ketone were isolated or prepared according to the procedure described by Dr. Hurley.³

All other reagents were used as obtained without further purification.

N-hexane (Northwest) was used as carrier for feeding experiments, tetradymol purification, and thin layer chromatography. Anhydrous diethyl ether (Baker) was also used for tetradymol purification and thin layer chromatography.

Ethanol, 95% and 100%, (US Industrial) was used as solvent for various solutions.

Potassium monophosphate, potassium diphosphate (Baker), and Tris (hydroxymethyl) aminomethane (Sigma) were used for

preparation of various buffers.

Compounds used as standard uncouplers were 2,4-dinitrophenol (2,4-DNP) (Eastman), and bis-hydroxy coumarin (dicoumarol) (Sigma).

Menthofuran (Eastman and benzofuran (Aldrich) were studied as possible uncouplers. Reduced tetradymol compounds, oxidized tetradymol and peperic acid were studied as obtained by Dr. Reeder² as possible uncouplers.

Oligomycin (Calbiochem) and dicyclohexylcarbodiimide (DCCI) (Aldrich) were used as inhibitors of ATPase.

Rotenone (K&K) and potassium cyanide (Baker) were used as Site I and III inhibitors, respectively, in various studies.

Sucrose (Baker), mannitol (Baker), ethylene diamine tetraacetic acid (EDTA) (Eastman) were used for isolation of mitochondria.

L-(+)-arginine HCl (Calbiochem), carbamyl phosphate (Sigma), L-(+)-ornithine (NBC), L-(+)-citrulline (NBC), aspartic acid (Calbiochem), urea (Baker), and arginosuccinic acid (Calbiochem) were used as standards and substrates in urea cycle experiments.

Nicotine adenine dinucleotide, reduced (NADH) (Calbiochem), α -ketoglutaric acid (Baker) and glutamic acid dehydrogenase (Calbiochem) were used in blood ammonia assays.

Adenosine-5'-diphosphate (ADP) (Sigma) was used as a substrate in oxygraph experiments. Adenosine-5'-triphosphate

(ATP) (Sigma was used as a substrate for various experiments.

Glucose (Baker), nicotine adenine dinucleotide phosphate (NADP) (Calbiochem), hexokinase (Sigma), and glucose-6-phosphate dehydrogenase (Calbiochem) were used for phosphate acceptor/oxygen ratio experiments. Sodium dodecyl sulfate (SDS) (MANN Research Lab.) was used as a standard detergent.

Nicotine adenine dinucleotide (NAD^+) (Calbiochem) was used as a substrate in reverse electron flow experiments.

Phospho(enol)pyruvate (Sigma) and pyruvate kinase (Sigma) were used for the ATP regenerating system in the ATPase experiments.

Bovine serum albumin (BSA) (Boehringer Mannheim) was used for the protein standard curve. Bovine serum albumin, recrystallized, lypholyzed, defatted (Sigma) was used for oxygraph experiments.

Trichloroacetic acid (Baker) and perchloric acid (Allied Chemical) were used to terminate various reactions.

N-carbamyl-L-glutamate (Sigma) was used for ammonia protection experiments with mice.

Chromerge (Manostat Corp) was used for development of thin layer chromatography plates.

t-butyl lithium (Aldrich) was used to make lithium salts in the pKa experiments.

II. Instruments

The Gilson Medical Electronic model KM oxygraph was used for all oxygen uptake studies with either mitochondria or submitochondrial particles.

Various spectral experiments were done on either the Cary 14 or the Varian Techtron Model 635 using matched quartz cells or, in the visible region, glass cells.

Dr. Callis' fluorimeter was used for mitochondrial reverse electron flow.

The Sorvall RC2-B centrifuge was used for mitochondrial isolation. The Beckman Model L-2 ultracentrifuge was used for isolation of submitochondrial particles.

The Potter-Elvehjem homogenizer was used in homogenizing all liver tissue.

The Bronwell Sic. Biosonik sonicator was used for sonication of mitochondria in the preparation of submitochondrial particles.

The New Brunswick Sci. Co., Inc. metabolite water bath shaker was used for various incubations.

III. Mice

Dub/ICR mice either purchased from Flow Laboratories or raised and maintained in our colony were used. The mice were housed in plywood and metal cages and maintained on Purina Lab chow and water "ad libitum".

In feeding experiments, tetradymol was administered via stomach tube using hexane as the carrier (4mg/0.1ml).

IV. Histology slides

Upon death or after sacrifice by decapitation small pieces of the liver were removed and placed in 10% neutral formalin obtained from Bozeman Deaconess Hospital. They were given to Gayle Callis for preparation of histological slides. All slides were stained with hematoxylin-eosin.

V. Isolation of mitochondria and submitochondrial particles

Mitochondria were isolated in 0.25M sucrose according to Schneider⁹⁶ for oxygraph work and in 0.25M sucrose in 1mM EDTA for all other mitochondrial work. Submitochondrial particles were isolated from mitochondria isolated in 0.25M sucrose in 1mM EDTA according to Kielly and Bronk⁹⁷ and suspended in either 0.3M phosphate buffer, pH 7.0, or 0.3M Tris-acetate buffer, pH 7.0, depending upon use.

VI. Standard determinations

A. Protein determination - Protein content of various preparations was determined by the method of Folin and Ciocolteau⁹⁸ using bovine serum albumin for the standard curve.

B. Inorganic phosphate assay - Inorganic phosphate was

assayed according to the method of Fiske and Subbarow as modified by Kaplan and del Carmen⁹⁹ using anhydrous monopotassium phosphate for the standard curve.

C. Urea assay - Urea was quantitated following the procedure of Brown¹⁰⁰ and a standard curve was first prepared.

D. Citrulline assay - Citrulline was quantitated by the color reaction of Archibald¹⁰¹ and a standard curve was prepared.

E. Ammonia assay - Ammonia was quantitated using the enzymatic procedure of Rubin, et al.¹⁰²

F. ATPase assay - ATPase activity was determined according to Pullman and Penefsky⁴⁸ for both whole mitochondria and sub-mitochondrial particles with the exception that with whole mitochondria 0.2ml of a 1.25M sucrose in 0.25M Tris-acetate buffer, pH 7.4, was used instead of 0.1ml of 0.5M Tris-acetate buffer, pH 7.4.

G. P/O - The phosphate acceptor/oxygen uptake (P/O) ratio was determined two ways for both mitochondria and submitochondrial particles. One procedure was directly from the oxygraph using the method of Chance and Williams.⁴⁵ The other was the measurement of ATP produced using the enzymatic method of Beyer.¹⁰³

H. Reverse electron flow - Reverse electron flow from succinate to NADH was determined for both mitochondria and submitochondrial particles following the procedure of Ernster and Lee.⁴⁹

I. Urea cycle activity - Ornithine transcarbamylase activity was determined using the procedure of Brown¹⁰⁴ with the modification that a 1:100 dilution of liver homogenate was used instead of a 1:1000 dilution.

VII. Experiments

Histology and toxicity - Mice were dosed with either 350 mg/kg tetradymol, 300mg/kg menthofuran, or 800mg/kg tetradymadiol monoisobutyrate. The mice dosed with tetradymol died in 7.1, 8.1, 9.3, 9.7, and 10.0 hours at which time their livers were removed. Histology slides revealed necrosis around the central vein being uniform in appearance with some vascular disruption and inflammation of the liver. Mice given tetradymadiol monoisobutyrate died in 8.75, 11.5, and 11.75 hours at which time their livers were removed. Histology slides revealed the same damage as with tetradymol except milder. Mice dosed with menthofuran died in 13.7, 13.7, 14.25, 13.0, and 12.8 hours at which times their livers were removed. Histology slides revealed similar damage as with tetradymol except it was much milder.

Clinical chemistry - a) Blood ammonia. Blood was removed by rectal orbital puncture from three mice and from three mice close to death (six hours) after having been given 350mg/kg tetradymol. The blood was allowed to coagulate then the serum

was used for blood ammonia assays. The three control mice gave values of 2.9, 3.0, and 4.0ug/ml serum; while, the drugged mice gave values of 11.4, 10.0, and 13.3ug/ml serum.

b) Blood chemistry - Ninety-six mice were divided into three control and three drugged groups of sixteen mice each. The three drugged groups were given 350mg/kg tetradymol. Blood was removed by rectal orbital puncture from all groups five to seven hours later. Blood from each group was pooled and allowed to coagulate then centrifuged to remove whole cells. At least 5ml of serum was taken off and frozen in test tubes. The six samples were then placed in dry ice and sent to Met Path in Hackensack, New Jersey where they conducted a blood screen of 24 tests. Results are given in Appendix A.

Urea Cycle Activity - The liver from one mouse was homogenized in 10 volumes (w/v) of distilled water, filtered through cheese cloth then diluted five fold for measurement of ornithine transcarbamylase activity which was described in Standard procedures. For each determination 0.1ml (0.44mg protein) was used (described earlier). Reactions were done at 25°C. Results are given in Table 28.

A mixture (0.5ml) containing 0.005M MgSO_4 , 0.02M ATP, 0.02M citrulline, 0.02M aspartic acid, and 0.05M phosphate buffer, pH 7.4, was placed in 12-3ml test tubes. The liver from one mouse

Table 28

Ornithine Transcarbamylase Activity

<u>Condition</u>	<u>umol Cit./mg P/hr</u>
Control	3.55, 3.80, 3.69
1mM Tetradymol	3.15, 3.59, 2.68
2mM Tetradymol	2.50, 2.36, 2.73
100uM 2,4-DNP	2.82, 2.80, 2.70

was homogenized in 10 volumes (w/v) of distilled water, filtered through cheese cloth and 0.5ml quantities (1.8mg protein) placed into each of the tubes. Blanks were prepared by adding 2ml 0.5M perchloric acid. The tubes were all centrifuged and 2ml of the supernatant used for the urea assay, which was described in Standard procedures. The results are in the following Table.

Table 29

Urea Production

<u>Conditions</u>	<u>umol/gm P/hr</u>
Control	382, 778, 546, 491
2mM Tetradymol	889, 491, 444, 656

Death Times - Twenty male mice were starved for eighteen hours and divided into four groups. The control group received 300ml/kg tetradymol. Another group received 300mg/kg tetradymol, then after 1.5 hours, 4 hours, and 6.5 hours 2mMole/kg glucose in water was administered intraperitoneally. The third group

was pretreated with 1mMole/kg each N-carbamyl-L-glutamate (NCG) and L-arginine 20 minutes before receiving 300mg/kg tetradymol and 4 hours after the tetradymol. The last group was pretreated with 2mMole/kg L-arginine 20 minutes before receiving 300mg/kg tetradymol and 4 hours after. The mice were observed and the time of their death recorded. The tetradymol control times were 7.1, 8.1, 9.3, 9.7, and 10 hours. The tetradymol plus glucose times were 8.7, 9.9, 11.7, 12.9, and 14.2 hours. The tetradymol plus NCG with arginine times were 8.0, 8.25, 12.75, 13.0, and 14.3 hours. The tetradymol plus arginine times were 7.9, 8.4, 12.9, and 13.1 hours (one died upon oral administration of tetradymol).

Effect of varying tetradymol concentration on state 4 respiration, respiratory control (RC) and phosphate acceptor to oxygen uptake ratio (P/O) - Mitochondria were isolated as described earlier. For every run 0.1ml of the mitochondrial suspension (1.15mg protein) was used with 1.87ml of incubation mixture consisting of 0.25M sucrose, 0.01M KCl, 0.005M $MgCl_2$, and 0.01M phosphate buffer, pH 7.4, 25 μ l of 384mM succinate, and 5 μ l of 8×10^{-4} M rotenone. Varying concentrations of tetradymol were prepared in 100% ethanol such that 5 μ l would result in the following final concentrations: 1, 5, 10, 25, 50, 100, 200, 350, and 500 μ M. Tetradymol was added after, a state 4, state 3, state 4 cycle on the oxygraph induced by

450nmol ADP, to observe any increases in state 4 respiration. Tetradymol was added before the ADP to observe changes in the P/O and RC ratios. All determinations were carried out at 25°C. The results of each determination are given in Table 30.

P/O in mitochondria and submitochondrial particles measured enzymatically - Mitochondria and submitochondrial particles were isolated as described before. An incubation mixture (1.7ml) containing 0.25M sucrose, 0.04M glucose, 0.01M phosphate buffer, pH 7.4, 0.0016M $MgCl_2$, and 3 units of hexokinase were placed in the oxygraph chamber. Then 0.2ml of the mitochondrial suspension (3.78mg protein) or 0.2ml submitochondrial particles suspension (1.44mg protein) was added along with 100ul of 384mM succinate and 5ul of $8 \times 10^{-4}M$ rotenone. For blanks, oxygen was allowed to be depleted, for control 25ul of 128mM ADP was added; otherwise, various effectors were added before the ADP and oxygen were allowed to go to completion. Then 1ml was removed and placed in a test tube containing 0.5ml of 0.5M perchloric acid and P/O values were determined as described in Standard procedures. For a standard, 750nmol of ATP was placed in the oxygraph chamber and carried through the experimental procedure. All determinations were carried out at 25°C. The results are tabulated in Table 31.

Reverse electron flow in mitochondria - A medium (3ml)

Table 30

Effect of Tetradymol on Mitochondrial
Oxidative-Phosphorylation

	Uncoupled rate State 4 <u>ngm-atom O/min</u>	RC	P/O
Control		4.1 4.5 5.1	1.88 1.91 1.96
1μM Tetradymol	0.00 0.00 0.00	4.6 4.0 5.1	1.89 1.90 1.96
5μM Tetradymol	0.00 0.00 0.00	4.4 4.5 4.6	1.86 1.89 1.95
10μM Tetradymol	8.85 14.75 5.90	4.8 4.8 3.7	1.89 1.90 1.94
25μM Tetradymol	23.60 20.65 8.85	3.5 3.7 3.4	1.84 1.90 1.96
50μM Tetradymol	26.55 26.55 20.65	3.8 3.2 2.7	1.85 1.89 1.90
100μM Tetradymol	44.25 70.80 59.00	2.38 2.13 2.00	1.55 1.61 1.70
200μM Tetradymol	118.00 109.20 56.00	1.12 1.17 1.38	0.80 0.84 0.88
350μM Tetradymol	98.00 108.00 118.00	1.00 1.00 1.00	0.00 0.00 0.00
500μM Tetradymol	99.40 94.40 75.5	1.00 1.00 1.00	0.00 0.00 0.00

Table 31

Mitochondrial P/O

<u>Condition</u>	<u>P/O</u>	<u>T test</u>
Control	1.30, 1.37	
0.5mM Tetradymol	2.03, 2.00	
1mM Tetradymol	1.02, 0.85	99.0
1.5mM Tetradymol	0.88, 0.85	99.9
2mM Tetradymol	0.68, 0.76	99.0
10μM Dicoumarol	0.80, 0.84	99.9
20μM Dicoumarol	0.44, 0.50	99.9
50μM 2,4-DNP	0.88, 0.93	99.9

consisting of 4mM succinate, 1.6mM KCN, 0.07M sucrose, 0.23M mannitol and 0.02M Tris-acetate, pH 7.4, was placed in a fluorimeter cell with 0.1ml of mitochondria (2.1mg protein) isolated as previously described with EDTA and 0.25M sucrose. The reaction was initiated by the addition of 50ul of 31.8mM ATP to give control rates. The NADH fluorescence was excited at 340nm and recorded at 460nm. Varying concentrations of tetradymol, 2,4-DNP, or oligomycin were added before the ATP and fluorescence monitored as before. All determinations were carried out at 24°C. The results are listed in Table 32.

Reverse electron flow in submitochondrial particles - A medium (3ml) containing 0.18M sucrose, 0.05M Tris-acetate buffer,

Table 32

Mitochondrial Reverse Electron Flow

<u>Condition</u>	<u>NADH Fluorescence</u> <u>Vertical sq/min</u> <u>(arbitrary units)</u>
Control	48.3, 38.6, 46.9
33uM Tetradymol	0.0, 0.0, 0.0
133uM Tetradymol	0.0, 0.0, 0.0
10uM 2,4-DNP	0.0, 0.0, 0.0
123uM Oligomycin	0.0, 0.0, 0.0

pH 7.5, 6mM MgSO_4 , 1.6mM KCN, 1mM NAD^+ , and 5mM succinate was placed in a quartz cell along with 25ul of submitochondrial particles (0.18mg protein). The reaction was started by the addition of 50ul of 180mM ATP and monitored at 340nm. Varying concentrations of tetradymol, dicoumarol, 2,4-DNP, oligomycin, or DCCI were added before the ATP and the reaction monitored as before. All determinations were carried out at 24°C. The rates of NADH production were recorded and the slopes used to calculate nmoles NADH/minutes. Results are shown in Table 33.

ATPase activity in mitochondria and submitochondrial particles - Mitochondria and submitochondrial particles were isolated as before. ATPase activity was measured as described previously. All determinations were carried out at 25°C. Results of these experiments are shown in Table 34 and 35.

Table 33

Submitochondrial Reverse Electron Flow

<u>Condition</u>	<u>nmoles NADH/ min</u>	<u>Condition</u>	<u>nmoles NADH/ min</u>
Control	84.0, 97.1, 90.3	33 μ M 2,4-DNP	26.9, 31.7
33 μ M Tetradymol	61.8, 64.9	66 μ M 2,4-DNP	20.6, 19.8
66 μ M Tetradymol	36.4, 38.0	133 μ M 2,4-DNP	0.0, 3.2
133 μ M Tetradymol	30.1, 33.3	33 μ M Dicoumarol	23.8, 25.3
244 μ M Tetradymol	14.3, 11.1	123 μ M Oligomycin	3.2, 3.2
		98 μ M DCCI	12.7, 26.9

Reversal of ATPase inhibition in mitochondria - Mitochondria were isolated as described previously. A medium (1.8ml) containing 0.25M sucrose, 0.01M KCl, 0.005M MgCl₂, and 0.01M phosphate buffer, pH 7.4, was placed in the oxygraph chamber with 0.2ml mitochondria (3.7mg protein), 25 μ l of 384mM succinate, and 5 μ l of 8×10^{-4} rotenone. Then either 5 μ l of 74.4mM oligomycin or 5 μ l of 59.4mM DCCI was added. ADP (25 μ l of 128mM) was added to make sure no increase in oxygen uptake occurred then uncouplers were added. All determinations were carried out at 25°C. Results are given in Table 36.

Effect of BSA on tetradymol uncoupling - A medium (1.7ml) containing 0.25M sucrose, 0.005M MgCl₂, 0.01M KCl, and 0.01M phosphate buffer, pH 7.4, was placed in the oxygraph chamber

Table 34

Mitochondrial ATPase

<u>Condition</u>	<u>mg Pi/ mg Protein/hr</u>
Control	1.26, 1.27
.5mM Tetradymol	1.58, 1.67
1mM Tetradymol	2.36, 2.47
2mM Tetradymol	4.89, 4.95
100uM 2,4-DNP	4.59, 4.63
200uM 2,4-DNP	4.10, 4.28

Mitochondrial ATPase

<u>Condition</u>	<u>mg Pi/ mg Protein/hr</u>
Control	0.33, 0.40
1mM Tetradymol	1.83, 2.32
1mM Tetradyma Monoisobutyrate	1.00, 0.80
1mM Menthofuran	1.46, 1.85
1mM Tetradyma Diol I	1.17, 1.04
1mM Tetradyma Diol II	0.84, 0.52
1mM Tetradyma Ketone	0.71, 0.46
1mM Oxidized Tetradymol	0.46, 0.56
1mM Reduced Tetradymol I	1.43, 1.30
1mM Reduced Tetradymol II	0.40, 0.39
1mM Reduced Tetradymol III	0.39, 0.45
1mM Benzofuran	0.55, 0.65
1mM Peperic acid	0.34, 0.37
200uM 2,4-DNP	2.83, 2.92

(structures of related compounds may be found in
appendix B)

Table 35

<u>Submitochondrial ATPase</u>	
<u>Condition</u>	<u>mg Pi/mg protein/hr</u>
Control	4.37, 3.70 5.00, 3.75
1mM Tetradymol	0.45, 0.88 0.14, 0.35
1mM Tetradymadiol Monoisobutyrate	3.04, 3.09 2.10
1mM Menthofuran	1.25, 1.25 1.30
200 μ M 2,4-DNP	5.84, 6.69 6.25, 5.80
1mM Tetradymol + 200 μ M 2,4-DNP	0.45, 1.55
1mM Menthofuran + 200 μ M 2,4-DNP	3.65, 3.60

along with 0.1ml mitochondria (2.15mg protein) isolated as before. Then 25 μ l of 384mM succinate and 5 μ l of 8×10^{-4} M rotenone were added. ADP (450nmol) was added after state 4 was achieved. Uncouplers were added and subsequently 0.2ml of a 10% solution of fatty acid free BSA. Alternatively, the BSA was added first then the uncouplers. Oxygen uptake rate was determined under each of the different conditions. All determinations were carried out at 25°C. Results are given in Table 37.

Effect of varying K⁺ on tetradymol uncoupling - Five

Table 36

Reversal by Tetradymol of DCCI
Inhibited Mitochondrial ATPase

<u>Condition</u>	<u>Rates</u> <u>ngm-atom O/min</u>
Control	81.5, 78.5, 76.6
200μM Tetradymol	117.9, 129.4, 120.5
Control	80.1, 75.6
10μM Dicoumarol	283.8, 265.0

Reversal by Tetradymol of Oligomycin
Inhibited Mitochondrial ATPase

<u>Condition</u>	<u>Rates</u> <u>ngm-atom O/min</u>
Control	90.5, 85.1, 88.0
200μM Tetradymol	93.9, 129.2, 129.2
400μM Tetradymol	173.2, 193.8, 187.8

different oxygraph reaction mixtures were prepared varying the K^+ concentration. All contained 0.25M sucrose, 0.005M $MgCl_2$, and 0.01M phosphate buffer, pH 7.4. The five concentrations of K^+ were 0mM, 5mM, 10mM, 15mM, and 20mM. Buffers (1.9ml) were placed in the oxygraph chamber then 0.1ml mitochondria (2.13mg protein), isolated as previously stated, then 25μl of 384mM succinate and 5μl of $8 \times 10^{-4}M$ rotenone, ADP (450nmoles) were added and respiration allowed to go from state 3 to state 4. Finally tetradymol was added to give a final concentration of

Table 37

Effect of BSA on Tetradymol Uncoupling

<u>Condition</u>	A	B	C
	<u>Unaffected ngm-atom O/ min</u>	<u>Uncoupled rate ngm- atom O/min</u>	<u>Rate after 1% BSA ngm- atom O/min</u>
200μM 2,4-DNP	66.9, 72.8	416.1, 404.5	66.9, 72.8
150μM Tetradymol	66.9, 64.0 69.8	113.5, 116.4 98.9	113.9, 119.3 133.9
200μM Tetradymol	64.0, 66.9	116.4, 122.2	136.8, 142.6
400μM Tetradymol	64.0, 69.8	244.4, 264.8	352.9, 360.8

<u>Condition</u>	Rate after 1% BSA ngm- atom O/min	D <u>Uncoupled ngm- atom O/min</u>
200μM Tetradymol	64.0, 62.8	64.0, 87.3
400μM Tetradymol	55.3, 61.1	133.9, 145.5

100μM. The increase in rates of respiration were then measured. All determinations were carried out at 25°C. Results are listed in Table 38.

Effect of varying pH on tetradymol uncoupling ability -

Six different oxygraph reaction mixtures were prepared by varying the pH. Each contained 0.25M sucrose, 0.01M KCl, 0.005M MgCl₂, and 0.01M phosphate buffer. 50ml portions were then adjusted with 1M HCl or 1M KOH to pH 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5. Succinate (25μl of 384mM) was also adjusted to the same pH. For each determination 1.9ml of one of the buffers

Table 38

Effect of Varying K^+ on Tetradymol Uncoupling

<u>Condition</u>	<u>Uncoupled rate State 4 ngm- atom O/min</u>
0mM K^+	32.0, 41.6 41.6, 51.2
5mM K^+	32.0, 28.8 41.6
10mM K^+	25.6, 38.4 44.8, 35.2
15mM K^+	28.8, 32.0 28.8
20mM K^+	32.0, 41.6 44.8

was placed in the oxygraph chamber along with the 25 μ l of succinate of the same pH. Then 0.1ml mitochondria (1.98mg protein) and 5 μ l of 8×10^{-4} rotenone. ADP (450nmol) was added and state 3 rates determined. In each case, after state 4 was obtained, tetradymol was added to give a concentration of 200 μ M and the rate determined. This procedure was followed for all six pH values. All determinations were carried out at 25°C. Results are shown in Table 39.

Effect of varying mitochondrial protein on uncoupling ability of tetradymol - Mitochondria were isolated as described earlier. Varying amounts of (0.05-3.0ml) mitochondria (1.07-6.42mg protein) were used. The reaction mixture contained

Table 39

Effect of Varying pH on Tetradymol Uncoupling

<u>pH</u>	<u>State 3 ngm-atom O/ min</u>	<u>State 4 ngm-atom O/ min</u>	<u>Uncoupled rate ngm-atom O/ min</u>
6.0	279.2, 267.9 273.5	81.1, 81.1 84.6	166.4, 163.6 166.4
6.5	358.1, 335.6 344.0	79.0, 76.1 79.0	205.9, 197.4 205.9
7.0	403.3, 403.7 386.3	79.0, 81.8 84.6	203.0, 225.6 253.8
7.5	414.5, 423.0 417.4	90.2, 90.2 87.4	180.5, 222.8 205.9
8.0	361.0, 330.0 344.0	79.0, 79.0 79.0	297.4, 220.0 169.2
8.5	273.5, 293.3 287.0	76.1, 76.1 79.0	222.8, 172.0 186.1

0.25M sucrose, 0.01M KCl, 0.005M MgCl₂, and 0.01M phosphate buffer, pH 7.4. An appropriate volume of this mixture was used such that with the mitochondria the total was 2ml which was placed in the oxygraph chamber. Then 25ul of 384mM succinate, 5ul of 8×10^{-4} M rotenone and 450nmoles of ADP were introduced. After state 4 resumed tetradymol was added to give a final concentration of 250uM and rates were recorded. All determinations were carried out at 25°C. Results are given in Table 40.

Effect of tetradymol, 2,4-DNP, and SDS on O.D. of a mitochondrial solution - A buffer (2.5ml) consisting of 0.25M

Table 40

Effect of Varying Mitochondrial Protein

<u>mg P</u>	<u>State 4 ngm-atom O/ min</u>	<u>Uncoupled ngm-atom O/ min</u>	<u>Difference</u>
1.07	9, 15, 15	51, 45, 54	42, 30, 39
1.82	42, 42, 45	102, 96, 102	60, 54, 57
2.14	39, 42, 51	99, 111, 110	60, 69, 69
2.68	57, 66	138, 147	81, 81
3.21	81, 81, 75	174, 174, 168	93, 93, 93
4.28	111, 111, 108	222, 216, 222	111, 105, 114
5.35	129, 141, 147	258, 240, 249	129, 99, 102
6.42	171, 174, 186	285, 300, 297	114, 126, 111

sucrose, 0.01M KCl, 0.005M MgCl₂, and 0.01M phosphate, pH 7.4 was placed in a ml quartz cuvette. Mitochondria (50ul) (0.54mg protein) was added. The optical density at 600nm was recorded. Either 25ul of 2,4-DNP for a final concentration of 25uM, 25ul of SDS for a final concentration of 100uM, 5ul of tetradymol in 100% EtOH for a final concentration of 100uM or 5ul of 100% EtOH were added and allowed to stand for five minutes before the optical density was recorded. The optical density of the buffer alone and buffer with each of the four conditions was obtained and subtracted from the final reading. All determinations were carried out at 25°C. Results are listed in Table 41.

Table 41

Effects on O.D. of a Mitochondrial Solution

<u>Condition</u>	<u>Optical Density Mitochondria</u>	<u>Optical Density Mitochondria and affector</u>
25uM 2,4-DNP	0.925, 1.000 1.010	0.905, 0.935 0.955
100uM SDS	0.960, 0.980 1.050	0.840, 0.850 0.910
100uM Tetradymol	0.940, 0.980 1.040	0.880, 0.955 0.970
.2% Ethanol	0.960, 0.960 1.010	0.925, 0.930 0.960

Table 42

SDS Effect on State 4

<u>Condition</u>	<u>State 4 ngm-atom O/ min</u>	<u>Uncoupled rate ngm-atom O/ min</u>
100uM SDS	64.0, 66.9	64.0, 66.9
400uM SDS	64.0, 61.0	116.4, 110.6
150uM Tetradymol	61.1, 51.3	116.4, 113.5

Table 43

Effect of Tetradymol on O₂ Uptake with Submito. Particles

<u>Condition</u>	<u>State 4 ngm-atom O/ min</u>	<u>ngm-atom O/ min after Tetradymol</u>	<u>After 10uM Dicumarol ngm-atom O/min</u>
250uM Tetradymol	248, 270, 236	202, 214, 186	254, 254, 251
500uM Tetradymol	260, 248, 264	153, 133, 124	152, 152, 143
750uM Tetradymol	273, 254, 236	65, 71, 62	112, 115, 105

The same conditions as described earlier to look at increase of state 4 respiration with tetradymol were used to look at increase of state 4 respiration with SDS. For each determination 1.2mg mitochondrial protein were used. All determinations were carried out at 25°C. Results are shown in Table 42.

Effect of tetradymol on O₂ uptake with submitochondrial particles - Submitochondrial particles were isolated as described earlier. A mixture (1.8ml) containing 0.25M sucrose, 0.01M KCl, 0.005M MgCl₂, and 0.01M phosphate buffer, pH 7.4, was placed in the oxygraph chamber along with 0.2ml submitochondrial particles (1.54mg protein). Twenty five microliters of 384mM succinate and 5µl of 8×10^{-4} M rotenone was added and the oxygen consumption rate determined. Various concentrations of tetradymol were added to give 250, 500, and 750µM and after sufficient time dicoumarol was added to give a final concentration of 10µM. All three rates were measured. All determinations were carried out at 25°C. Results are shown in Table 43.

Uncoupling ability of tetradymol-like compounds - The same conditions as described earlier to look at increases of state 4 respiration rate with tetradymol were used in these experiments. Mitochondrial protein (3.6mg) were used for each determination. All determinations were carried out at 25°C. Results are given in Table 44.

Table 44

Uncoupling Ability of Tetradymol Like Compounds

Condition 500uM each	Uncoupled Rate State 4 <u>ngm-atom O/min</u>
Tetradymol	300, 306
Tetradymadiol Monoisobutyrate	192, 192
Tetradyma Diol I	33, 39
Tetradyma Diol II	4.5, 4.5
Tetradyma Ketone	15, 15
Tetradymol Reduced I	51, 57
Tetradymol Reduced II	69, 63
Tetradymol Reduced III	63, 63
Oxidized Tetradymol	24, 33
Menthofuran	366, 312
Benzofuran	33, 33
Peperic acid	15, 15

Effect of tetradymol on the three phosphorylation sites -

Mitochondria were isolated as described previously. For the three coupling sites 1.8ml of the same reaction mixture containing 0.25M sucrose, 0.01M KCl, 0.005M $MgCl_2$, and 0.01M phosphate buffer, pH 7.4, was used and 0.2ml mitochondria (1.9mg protein). For site I 25ul of 384mM glutamate was used. For site II 25ul of 384mM succinate and 5ul of 8×10^{-4} M rotenone were used. For

site II 25 μ l of 384mM ascorbate, 10 μ l of 27mM TMPD, and 5 μ l of 8×10^{-4} M rotenone were used. Tetradymol concentrations of 200 μ M and 350 μ M were used to inspect the relative uncoupling of each site. ADP (450nmoles) was added and RC and P/O values were determined as described previously and compared to control values. All determinations were done at 25°C. Results are listed in Table 45.

Determination of the pKa of tetradymol - The basic spectrophotometer procedure of McEwen was followed. Both hexane and ether were redistilled from lithium aluminium hydride before use. All preparative operations were carried out under nitrogen purified by being passed through a column of BSTA catalyst and anhydrous calcium chloride. All spectral determinations were carried out in sealed quartz cuvettes. A 32.4 μ M solution of 4-chloro-2-nitroaniline in ether resulted in peaks at 403m μ ($A=.1889$, $\epsilon=4.7 \times 10^3$) and 234m μ ($A=.755$, $\epsilon=2.29 \times 10^4$). To make the salt of 4-chloro-2-nitroaniline, 21.6mg (.125nmoles) of 4-chloro-2-nitroaniline were placed in a 50ml two neck round bottom flask. One neck was sealed with a rubber septum and the other connected to a two way stopcock. Those two parts of the apparatus were connected to a three way stopcock, one way allowed evacuation with an aspirator and the other permitted entry of purified nitrogen. The apparatus was then evacuated and filled with nitrogen four times. Then 10ml of

Table 45

Relative Uncoupling at 3 Sites

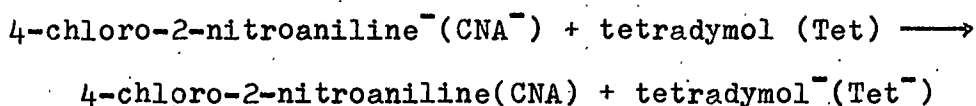
<u>Condition</u>	<u>Glutamate</u>			<u>P/O</u>		
	<u>RC</u>					
Control	3.71, 3.38, 3.93			3.13, 2.57, 2.74		
	3.72, 4.07			2.42, 2.38, 3.38		
				2.57		
200µM Tetradymol	1.22, 1.32, 1.62			2.22, 2.12, 1.99		
350µM Tetradymol	1.00, 1.00, 1.00			0	0	0

<u>Condition</u>	<u>Succinate</u>					
Control	5.61, 5.45, 5.14			1.77, 1.81, 1.84		
	5.33, 4.91, 4.86			1.84, 1.90, 1.92		
	6.32, 5.36			1.79, 1.72		
200µM Tetradymol	1.63, 2.38, 2.12			1.34, 1.48, 1.45		
	2.16			1.45		
350µM Tetradymol	1.00, 1.00, 1.00			0	0	0

<u>Condition</u>	<u>Ascorbate/TMPD</u>					
Control	1.44, 1.34, 1.31			0.59, 0.80, 0.58		
	1.34, 1.26, 1.41			0.73, 0.64, 0.71		
	1.26, 1.42			0.66, 0.71		
200µM Tetradymol	1.09, 1.19, 1.10			0.41, 0.58, 0.49		
350µM Tetradymol	1.00, 1.00, 1.00			0	0	0

hexane and 0.1ml (.123mmoles) of 1.23M t-butyl lithium were introduced through the rubbered septum using syringes. After 10 minutes the apparatus was evacuated to remove hexane and 2-methyl propane. The two way stopcock was then closed and transferred with the round bottom flask to a dry bag containing

anhydrous calcium chloride, ether, and all glassware necessary to accomplish all transfers. The dry bag was sealed then evacuated and filled with purified nitrogen four times. Ether (25ml) was added to the round bottom flask and a 50 fold dilution with ether made of that solution. Three milliliter aliquots were transferred to three cuvettes. One cuvette contained 5 μ l (0.3 μ moles) of a 60 μ M solution of tetradymol in ether. After removal from the dry bag one drop of water was added to one cuvette to convert the salt back to the acid form of 4-chloro-2-nitroaniline. A spectrum of the salt using the acid in the reference beam resulted in a peak at 318m μ (A=0.36) and a trough at 405m μ (A=0.06). The spectrum of the salt with tetradymol using the acid in the reference beam resulted in a peak at 318m μ (A=0.28) and a trough at 405m μ (A=0.0). A rough calculation of the k_{eq} was made for the following reactions:

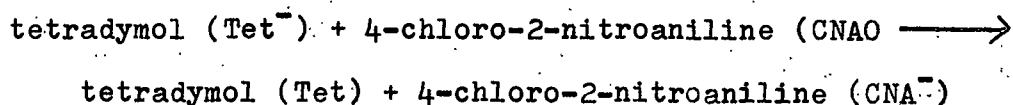


Resulted in:

$$k_{eq} = \frac{(\text{CNA})(\text{Tet}^-)}{(\text{CNA}^-)(\text{Tet})} = \frac{(0.07\mu\text{M})(0.07\mu\text{M})}{(0.93\mu\text{M})(0.43\mu\text{M})} = 5.7 \times 10^{-3}$$

The converse experiment (first making the salt of tetradymol) was done by reacting 44.56mg (0.19mmoles) of tetradymol with 0.15ml (0.18mmoles) of 1.23M t-butyl lithium. The same

dilutions were carried out in the dry bag. A 3ml aliquot of the tetradymol salt was reacted with 0.45 μ moles of 4-chloro-2-nitroaniline in one cuvette while 0.45 μ moles of 4-chloro-2-nitroaniline was placed in another cuvette with 3ml ether. A spectrum of the tetradymol salt with 4-chloro-2-nitroaniline using 4-chloro-2-nitroaniline in the reference beam resulted in $A=0.53$ at 318 μ and $A=0.12$ at 405 μ . A rough calculation of the k_{eq} was made for the following reaction:



Resulted in:

$$k_{eq} = \frac{(\text{Tet})(\text{CNA}^{-})}{(\text{Tet}^{-})(\text{CNA})} = \frac{(1.37\mu\text{M})(1.37\mu\text{M})}{(0.13\mu\text{M})(0.13\mu\text{M})} = .94$$

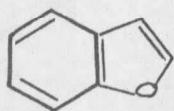
APPENDIX A
Clinical Tests

CLINICAL TESTS

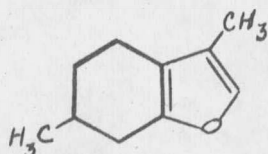
Test	Units	1	2	3	1	2	3
		(Normal)			(Drugged)		
Ca	MG%	9.6	9.4	10.0	10.4	9.6	9.9
Ph	MG%	8.7	7.4	7.9	8.9	9.7	9.5
BUN	MG%	24.0	23.0	19.0	25.0	31.0	24.0
Creat	MG%	.6	.6	.4	.8	.3	1.0
BUN/Creat		40.0	38.33	47.5	31.25	103.33	24.0
Glucose	MG%	163.0	88.0	84.0	78.0	54.0	63.0
T. Prot.	GM%	5.9	5.8	6.3	6.8	5.8	6.8
Alb.	GM%	3.4	2.9	3.2	3.7	3.0	3.2
A/G		1.36	1.00	1.00	1.19	1.07	.89
Thy. Turb.	Units	2.00	1.00	2.00	2.00	1.00	2.00
Tot. Bili.	MG%	.32	.17	.17	.69	1.06	.59
Dir. Bili.	MG%	.01	.01	.01	.01	.01	.01
SGPT	Units	227	342	291	227	392	392
SGPT	Units	143	130	117	161	433	449
Alk. Phos.	Units	15.5	42	46	23	72	87
LDH	Units	1562	967	1082	1541	1116	1179
Amylase	Units	92	133	141	90	162	136
Cholest.	MG%	205	120	144	233	125	162
Beta Lip.	Units	5.9	8.1	10.0	6.2	5.0	7.1
T. Lip.	GM%	.33	.51	.62	.47	.48	.59
Sodium	MEQ/L	155	145	155	149	151	149
Potas.	MEQ/L	5.9	6.2	8.4	8.5	10.1	11.8
Cl	MEQ/L	120	113	116	118	119	124
Uric Acid	MG%	3.6	3.7	5.6	3.9	1.5	3.7

APPENDIX B

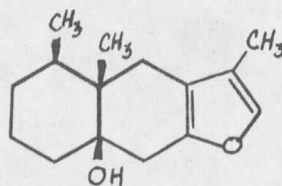
Structures of Related Compounds



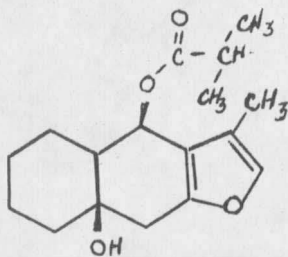
Benzofuran
 $R_f = .548$



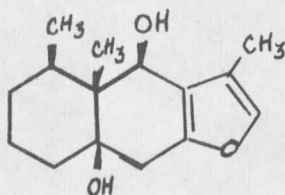
Menthofuran
 $R_f = .65$



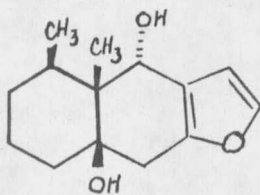
Tetradymol
 $R_f = .49$



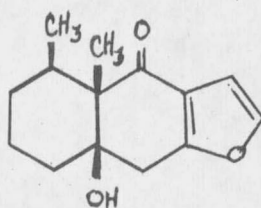
Tetradymadiol
Monoisobutyrate
 $R_f = .54$



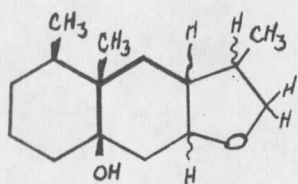
Tetradymadiol I
 $R_f = .301$



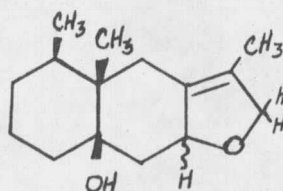
Tetradymadiol II
 $R_f = .41$



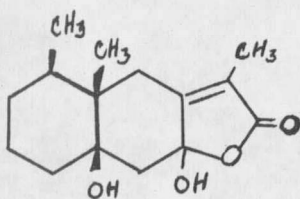
Tetradymaketone
 $R_f = .36$



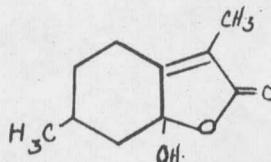
Reduced Tetradymol I + II
 $R_f = .46, .307$, respectively



Reduced Tetradymol III
 $R_f = .301$



Oxidized Tetradymol
 $R_f = 0$



Peperic Acid
 (Oxidized Menthofuran)
 $R_f = 0$

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Paradoxical uncoupling
activity of tetradymol

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