

## Fractionation of Coal Extracts with Bacterial Enzymes.

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**ABSTRACT:** Common and novel bacterial enzymes or *de novo* synthesized unique organic ethers reacted with Coal extracts yielding coal breakdown products. The diphenyl ether was synthesized *de novo* from methyl benzoate and 1-Diphenyl-1,3-butanedione preparation of the di-cation of butanedione with the aid of sodium hydride (NaH). After synthesis of 2,6-Diphenyl-4-methoxypyridine and other less common ethers, dissolution of coal fractions yielded various levels of carbon fragments. Ether extraction of Coal (Illinois No. 6) resulted in release of aromatic and short chain fractions. Enzymatic dissolution of Coal and ether extracts also yielded identifiable carbon fragments. Specific enzymatic dissolution of Coal with pure flavin containing enzymes remains a goal. This approach involves: 1-more complete kinetic characterization of the proposed NADPH Reductase/comproportionation system, 2-initial experimentation into flavodoxin components common to both the Nitrogenase system and the electron transport system of *Azotobacter vinelandii*, 3-the degree of complementarity between these systems judged by component interaction and immunologic cross reactivity.

**INTRODUCTION:** *De novo* synthesized ethers reacted with Coal Extracts yielding breakdown products (1). A novel pyridine ether (I) synthesized *de novo* from 1-Phenyl-1,3-butanedione and methyl benzoate and two more common pyridine ethers (II & III) solubilized dried Coal Extracts (Fig.1).

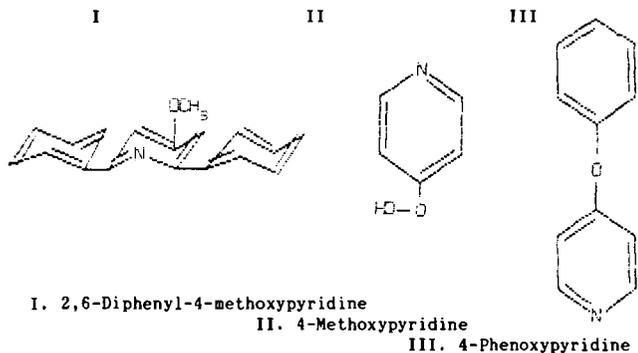


Fig 1. Organic Structures

The purpose of these experiments, in general, is to differentiate the ultrastructure from that of living structure such as the bacterial spore. In particular, unlike bacterial spores that possess easily discernible pore sizes, coal is much more difficult to approximate because of its local regions of heterogeneity. That is unlike bacterial spores, different regions of the coal ultrastructure apparently differ markedly from other domains of the ultrastructure. Interaction of the pyridine ethers with the coal structure can considerably curtail interpretation. However, the study reported above

describing the abbreviated exposure of 4-methoxypyridine pyridine to coal assumes limited if not exclusionary chemical reaction with the coal. The ultrastructure of coals is susceptible to study using small molecular weight organic molecules that are not susceptible to alternation by the interior of the coal pores. In these experiments commonly accepted organic molecules that are very resistant to alternation in such organic milieu, ethers. Theoretically these molecules can be used as molecular sieves to approximate the size of coal pores. Unfortunately, the pyridine ethers are somewhat unreasonable solvent because they react with the coal ultrastructure after prolonged periods of time. Generally, however, the ethers and the enynes reactions with the coal were understandable and provided valuable data.

#### RESULTS:

*1,5-Diphenyl-1,3,5-pentanetrione* 1,5-Diphenyl-1,3,5-pentanetrione, the enolate carbanion of 1-phenyl-1,3-butanedione, formed after proton extraction with sodium hydride in the aprotic solvent, 1,2-dimethoxy ethane (monoglyme). Condensation of this carbanion with methyl benzoate yielded the triketone, 1,5-diphenyl-1,3,5-pentanetrione. This aryolation apparently involves the di-anion of the B-diketone which forms in a

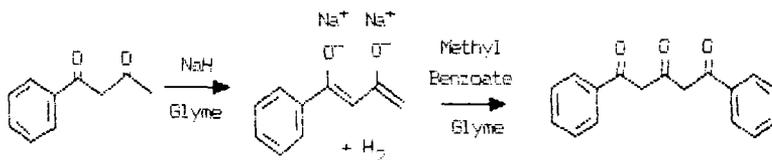


Fig. 2. Synthesis of 1,5-Diphenyl-1,3,5-pentanetrione

"step-wise" extraction of its  $\alpha$ -hydrogens. The terminal  $\alpha$ -hydrogen is not the first extracted from the b-diketone, but is only extracted after mono-anion formation and in the presence of methyl benzoate. The terminal  $\alpha$ -hydrogen is, however, the site of reaction of the b-diketone with the carbonyl carbon of the ester. The methoxide group of the ester, neutralized by the Na<sup>+</sup> counter ions, then leaves the condensation product. Exposure to air with its incipient moisture and neutralization of the reaction mixture with 6N HCl provide the protons for neutralization of the carbanions present and formation of the triketone.

This reaction occurs with a high degree of efficiency yielding 80-90% product (86.9%). Apparently once formed, the di-anion is quite reactive and the reaction is evidently highly selective for the terminal carbon of the dianion. As expected, the symmetric triketone formed, was soluble in diethyl ether, but its solubility varied considerably depending on the pH of the reaction mixture.

These differences could be a reflection of minor changes in the structure of the triketone. The spectral data substantiates that they are not reflective of impurities present. Variation of the color, crystalline form and melting points of the triketone which substantiate similar earlier observations are also suggestive of small variations in the structure of the triketone. Because of the H-bonding, various keto-enol tautomeric forms can be envisioned and as pH changes occur in the solvent all of these forms are undoubtedly present in the solution. At low pH values the triketone is probably found as a doubly H-bonded enolic structure (Formula 4), at intermediate pH values as a mono-H-bonded structure (Formula 2) and at high pH values as a keto form (Formula 1) with little H-bonding.

*2,6-Diphenyl-4H-pyran-4-one* The triketone can be protonated in concentrated sulfuric acid in the 1-or 5-keto position leading to the formation of enolic

triketones. The triketone is then cyclized by the formation of an intramolecular acetal. Upon dehydration the pyran is completed yielding the 2,6-

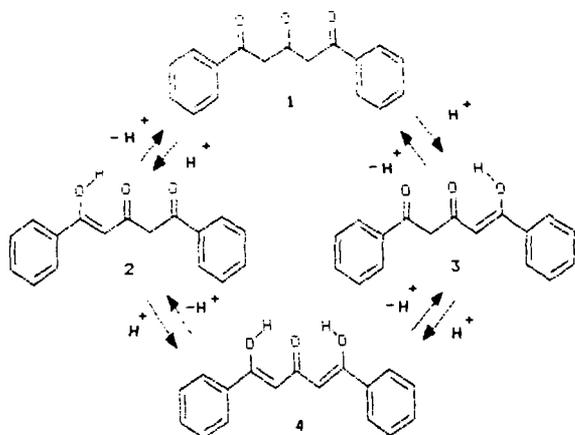


Fig. 3. Hydrogen Bonding of 1,5-Diphenyl-1,3,5-pentanetrione

diphenyl-4H-pyran-4-one. The reaction occurs with great efficiency (89-91%) suggesting that the stability of the 6-membered ring in the product is the selecting factor in the formation of the pyran derivative.

**2,6-Diphenyl-4(1H)-pyridinone** This pyridinone was produced by the cyclization of the 1,5-diphenyl-1,3,5-pentanetrione with liquid ammonia. Because of the high pH of the reaction milieu, which causes the enolization of the ketone groups, the addition product is the primary amine-enol or dienol intermediate.

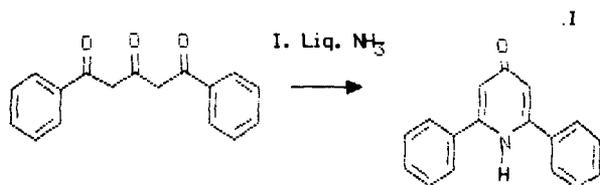


Fig. 4. Synthesis of 2,6-Diphenyl-4(H)-pyridinone

Cyclization by addition of the primary amine to the other terminal carbon of the pentanetrione chain then readily occurs (Table 1).

This di- or trienolic structure is then dehydrated to form the highly conjugated diphenyl-pyridine derivative with its quinoid-like pyridine ring. Noteworthy in this synthesis is the modification of the original synthetic procedure by addition of the dehydrating agent, sodium sulfate, to the reaction milieu. The combination of the anhydrous ethanol and sodium sulfate promoted the reversible dehydration of the product and gave an increase in yields of 32.3-35.4 to 66.6-75.1%.

Table 1. Physical Properties of Synthetically Prepared Precursors and Ethers

This addition had little effect on the physical properties of the product. The yields were also increased from a literature value of 45% to an average value of 70% in our experiments. Also, sodium sulfate added in only the second step was equally effective. In the presence of this additional dehydrating agent, the efficiency of the reaction is quite high. Because the product pyridinone is highly conjugated investigators have obtained its UV spectrum. This property and the electronic configuration around the heterocyclic nitrogen, suggest that the molecule may be



Fig. 5. 2,6-Diphenyl-4-Methoxypyridine (A)

planar even though this would involve an eclipsed conformation of the phenyl rings (A) and the pyridinone. Steric hindrance would not preclude this possibility, since the phenyl rings could move freely about the carbon to connecting them to the heterocyclic ring. The heterocyclic ring is of a quinoid not benzenoid configuration which disallows enol formation of the keto group in the C-4 position. The highly conjugated nature of the pyridinone allows little stereochemical analysis of the molecule which contains no asymmetric carbon, but the presence of two chiral centers during the closure-dehydration mechanism suggests specificity of the protons lost from the C-3 and C-5 positions as dehydration occurs. The formation of the double peak feature at 3090 and 3140  $\text{cm}^{-1}$  not found in the original triketone, is evidence of the presence of the heterocyclic ring. This coincides with the loss of some of the carbonyl intensity and features. This is also strongly suggested by the loss of IR features from 900-1299  $\text{cm}^{-1}$ . The NMR spectrum also reveals the downfield migration of the non-aromatic protons of the triketone to the C-3 and C-5 protons of the heterocyclic ring. The signal for these protons occurred within the signal for the three terminal protons of each phenyl ring of the pyridinone. It could be observed when using a sweep width of 250 instead of 500 Hz. The proton of the heterocyclic nitrogen was not observable even at a chemical shift of 15 ppm. Mass spectral analysis revealed a molecular peak at 247 me units and the usual fragments associated with the molecule. The synthesized pyridinone possessed the literature values of the physical properties. In addition, the physical properties of the triketone and pyran were considerably different. These results suggest because of their interdependence that the structural parameters attributed to these isolates are consistent and reputable.

#### *Enzymatic Properties*

Enzymological Purification of NADPH:Flavodoxin Oxidoreductase Ten to fifteen mgs of purified reductase remained after purification of 220 times from the 250,000 x g supernatant. Gel filtration of the FLD reductase succeeds only on large diameter pore size gels, i.e., Sepharose 2B or 4B or Ultragel-32. This suggests either a very large molecular weight enzyme, complexation of the enzyme with other proteins, or polymerization of a small molecular weight enzyme into large aggregates. Spectra of the flavin group of the enzyme undergo reduction by light and EDTA. The absorption spectrum of Flavodoxin Oxidoreductase that contains a FAD residue as a prosthetic group is sensitive to EDTA reduction in light.

Enzymological Properties Purified FLD reductase shows little selectivity of substrates and reaction rates appear entirely dependent on the redox potential of the substrate. Progress curves of FLDH<sub>2</sub> formation are biphasic and suggest an initial rapid rate of FLDH<sub>2</sub> formation (0-2 ms) and a second more persistent FLDH<sub>2</sub> formation (2-12 ms) (Table 2). Double reciprocal plots of either rates are straight lines over a limited range of substrates concentration (15-50 mM FLD<sub>AV</sub>). The initial rate of formation of FLDH<sub>2</sub> resides within the reduction rate range described for the other electron acceptors. The K<sub>m</sub> of FLDH<sub>2</sub> formation is also quite comparable to that of the other electron acceptors (Table 2). The change of K<sub>m</sub> at 2 ms

Table 2. Enzyme Parameters and Kinetic Constants of the Enzymatic Reduction/Comproportionation of FLD<sub>AV</sub> and the Me-FLD<sub>AV</sub>.

of K<sub>m</sub> at 2 ms is consistent with the idea that the affinity of the FLD for the FLD reductase changes as the FLDH<sup>\*</sup> concentration increases. If this is the operative mechanism of FLD<sub>AV</sub> reduction that is quite consistent with the fluorescent data, it suggests that NADH reduces FLD then the FLDH<sub>2</sub>-NAD<sup>+</sup> complex reduces FLD<sub>AV</sub>.

#### Enzymological Reactivity

Spectrophotometric Assay: The enzymatic reduction of both FLD and N<sub>3</sub>-CH<sub>3</sub>-FLD straight forwardly demonstrates the primary formation of FLDH<sup>\*</sup> and its analogue. The initial velocity (v<sub>0</sub>) was 10% larger for substituted flavodoxin indicating that it is a better substrate. The formation of N<sub>3</sub>-CH<sub>3</sub>-FLDH<sup>\*</sup> also remains more sustained producing 32% of the possible N<sub>3</sub>-CH<sub>3</sub>-FLDH<sup>\*</sup> in the first 10 ms of reduction. During the same period 16% FLDH<sup>\*</sup> undergoes reduction. Second order rate equations facilitated quantitation of these kinetic differences.

Product Formed Graphic representation of the hydroquinone formation (Reaction 1) exhibits a shift from one constant (k<sub>1A</sub>) to another (k<sub>2A</sub>) as the reaction proceeds. The duration of the initial kinetic parameters varies greatly from native to substituted flavodoxin. The modification of the initial rate (slope) occurs more quickly and completely with FLD. This modification accelerated by ionic strength, causes further displacement of the 5 mM Tes, line toward the x-axis at 25 mM Tes. Contrariwise, these ionic strengths don't affect the secondary kinetic constants because the slopes remain constant. The dFLDH<sup>\*</sup>/dti is about as large at 25 mM Tes. Its formation is linear throughout the observed reduction.

Enzymological Properties of NADH:Ferricyanide Dehydrogenase: Topological and ultrastructural membrane features accrued during comparison of walled, *Staphylococcus aureus*, and wall-less, *Acholeplasma laidlawii*, bacterial membranes. Osmotic lysis of protoplasts of these organisms produced membrane vesicles or large membrane fragments, but alumina grinding or sonication of these protoplasts or cells produced much smaller particles whose morphologic origin was difficult to determine. Disrupted membranes from either organism were covered with ribosomes from 15 to 30 nm diameter adhering almost exclusively to the inside of the vesicles or fragments.

An NADH dehydrogenase possessing a specific activity 3-5 times that of membrane bound enzymes was obtained by extraction of *A. laidlawii* membranes with 9.0% ethanol at 43° C. This dehydrogenase contained only trace amounts of iron (suggesting an uncoupled respiration), a flavin ratio of 1:2 FAD to FMN, and 30-40% lipid, which could explain its resistance to sedimentation. It efficiently utilized ferricyanide, menadione and dichlorophenol indophenol as electron acceptors, but not O<sub>2</sub>, ubiquinone Q10 or cytochrome c. Lineweaver-Burke plots of the dehydrogenase were altered to linear plots of the dehydrogenase were altered to linear functions upon extraction with 9% ethanol. In comparison to other respiratory chain-linked NADH dehydrogenases in cytochrome containing respiratory chains, this dehydrogenase was characterized by similar K<sub>m</sub>'s with ferricyanide, dichlorophenol indophenol, menadione as electron acceptors, but considerably smaller V<sub>max</sub>'s with ferricyanide, dichlorophenol indophenol, menadione as electron acceptors, and smaller specific activities. Some kinetic properties of the dehydrogenation, the uniquely high glycolipid content and apparently uncoupled respiration at Site I characterized this NADH dehydrogenase from this truncated respiratory chain.

#### DISCUSSION:

Studies revealed hydrogen bond activation of alkyl-aryl ethers (I) toward cleavage by formation of its mono- then di-anion and combination with methyl-benzoate. These reactions provide good yields due to the driving force of the charged molecular species. Our yields provide excellent conditions for continued synthesis of ether precursors. Unfortunately, the inefficiency of the diphenyl-chloropyridine and the phenyl-ether synthetic steps limited the total yields of ethers.

Current thinking now suggests that coal is an elastomer in which strong internal hydrogen bonding gives it high internal glass transition temperatures. The same acidic hydrogen donors and basic hydrogen receptors that provide strong hydrogen bonds in the bulk structure populate the surface of coal powders. A dominant property of this molecule is the H-bonding capacity of the 2,6-diphenyl-1,3,5-pentanetrione. A variety of physical properties reflected this dominating molecular effect during isolation procedures.

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**SUMMARY:**

1,5-Diphenyl-1,3,5-pentanetrione was cyclized to form 2,6-Diphenyl-4(1H)-pyridinone with liquid ammonia. The resulting ether, 2,6-Diphenyl-4-methoxypyridine, from substitution of the keto (C-4 position) of the pyridinone, was used to extract Coal. Several extraction treatments of Coal based upon enzymatic treatment of extracted coal are foreseen.

**Table 1. Physical Properties of Synthetically Prepared Precursors and Ethers**

Name	Recrystallization Solvent	Color	Mp (Rp)	% Yd
1,5-Diphenyl-1,3,5-pentanetrione	95% Ethanol	Yellow	107-109	57.7
2,6-Diphenyl-4(1H)-pyridinone	Benzene	Cream	176-179	66.6
2,6-Diphenyl-4-chloropyridine	95% Ethanol	White	83-84	86.3
2,6-Diphenyl-4-methoxypyridine	Methanol	White	79-80	46.3
4-Methoxypyridine	Distilled Water	Clear	41-45	
4-Phenoxypyridine	Distilled Water	Clear	78-79	

**Table 2. Enzyme Parameters and Kinetic Constants of the Enzymatic Reduction/Comproportionation of  $FLD_{AV}$  and the  $Me-FLD_{AV}$ .**

Properties	FLDH		$N_3-CH_3-FLDH$	
	5 mM Tes pH=7.4	25 mM Tes pH=7.4	5 mM Tes pH=7.4	50 mM Tris pH=9.5
$(FLDH^+ / FLDH_2)$ of Final Product	$\frac{10.55}{4.06} \mu M$	$\frac{12.75}{1.70}$		
$dFLDH_2/dt_i = v_0$	0.53 nmol	0.33	0.58	0.26
$dFLDH^+ / dt_i$	0.10	0.15	0.58	0.26
$k_{1a}$	$8.56 M^{-1} sec^{-1}$	5.86	8.04	2.89
$k_{1b}$	$2.75 M^{-1} sec^{-1}$	1.94	3.34	1.52
$k_2^*$	$60.60 M^{-1} sec^{-1}$	318.00	31.37	30.00
$K_{sq}$	$0.10 M^{-1} sec^{-1}$	1.60	0.39	0.34