THE PHOTOCHEMISTRY AND BIOCHEMISTRY
OF AROMATIC N-OXIDES

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(Ph. D. Thesis)

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On the seventh day God rested

And God put man on the Earth and said to him -- you are the ruler of all this

Oh, God, let me be guided correctly

I look at the universe and I am in awe. A gift that I may even see what my eyes see, hear what my ears hear, feel what my body feels. And all that I see submits to me even the far reaches of space -- but though I can examine and change and mold, yet all is creation and the basic nature does not change.

Your universe is vast -- I can see but a small piece of it. And I can understand but a little of it. May I submit and be guided correctly. And as I grasp the nature of things and use them, I see that all must answer to the God. For, indeed, this Earth is ruled by man, and man must answer to God for his uses. The burden of a ruler is vast, as vast as the subjects. For, if the ruler is unjust and goes contrary to the nature of his subjects, his kingdom will be torn apart. O, Lord, forgive us for our pride and selfishness.

I came to Berkeley, and I shall leave it to itself.

Oh, Lord, may I use my intellect so that some of the problems of living here can be solved. May I do my work without jealousy, nor put it to unjust use. Guide me so that my people shall be free.

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THE PHOTOCHEMISTRY AND BIOCHEMISTRY OF AROMATIC N-OXIDES

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ABSTRACT

The photochemistry of pyridine N-oxide and nicotinamide N-oxide was studied in various solvents. It was found that pyridine N-oxide reacts in two ways when irradiated: 1) rearrangement and 2) loss of oxygen. Irradiation of pyridine N-oxide in cyclohexene gave, besides the rearranged products, oxygenated cyclohexene compounds (cyclohexene oxide, cyclohexanol, and cyclohexanone). This oxygen transfer reaction served as a good model for the activation of molecular oxygen by mixed function oxidases.

The possibility that an aromatic N-oxide, nicotinamide N-oxide, might act as a discrete intermediate in the activation of molecular oxygen by mixed function oxidases was explored. The enzyme systems studied were the squalene epoxidase, laurate hydroxylase, and p-hydroxybenzoate hydroxylase. Nicotinamide N-oxide does not act as an oxygen source for these mixed function oxidases.

GENERAL INTRODUCTION

General Introduction

Since the discovery of oxygenase systems, the least understood step has been the incorporation of an oxygen atom into a substrate from molecular oxygen. This step has been referred to as the "oxygen activation" step. The topic of this thesis is the investigation of cofactors involved in oxygen activation. The particular possible cofactor studied is nicotinamide N-oxide. Such a cofactor would fit into the following general scheme for "mixed function" oxidases:

Several lines of evidence suggest nicotinamide N-oxide as a cofactor in oxygen activation.

1. Direct oxygen transfer (determined by isotopic labeling) from nicotinamide N-oxide to xanthine by xanthine oxidase:

2. Increase in activity of enzyme systems (such as hydroxylation and oxidative dealkylation) in liver microsomes with the addition of nicotinamide. (These reactions also require TPNH and O_2 .)

3. Experimentally determined production of nicotinamide N-oxide from nicotinamide by rat liver homogenates.

4. By analogy to a model system: the photochemical transfer of oxygen from pyridine N-oxide to cyclohexene. The photochemical transfer from pyridine N-oxide to aromatic substrates has reproduced the "NIH shift". (The NIH shift is a property of hydroxylase systems.) The postulated photochemical intermediate in the transfer of oxygen could be the same intermediate produced by hydroxylase.

The experimental approach employed in testing the hypothesis that nicotinamide N-oxide could act as a general cofactor in mixed function oxidase systems was as follows: Under anaerobic conditions, nicotinamide N-oxide was introduced into the enzyme system instead of oxygen. The presence or absence of oxygenated substrate was then determined. Three enzyme systems were used--squalene oxidase and fatty acid hydroxylase in rat liver homogenates, and purified p-hydroxy benzoic acid hydroxylase from pseudomonas. In each case nicotinamide N-oxide did not act as an oxygen donor. Therefore the transfer of oxygen from nicotinamide N-oxide in the xanthine oxidase system cannot be generalized to mixed function oxidase systems. However, the function of nicotinamide in microsomal oxidations remains uncertain.

This thesis will be divided into two parts: The first will deal with the photochemistry of aromatic N-oxides (a photochemical model for hydroxylase systems is developed); and the second will deal with the biochemistry of nicotinamide and nicotinamide N-oxide. Failure of the nicotinamide N-oxide to act as a general oxygen transfer agent is demonstrated.

Instrumentation

The various instruments used throughout my thesis work are described below.

Infrared spectra (IR) were taken on a Perkin Elmer 257 instrument. Ultraviolet absorption spectra (UV) were taken on a Cary 14. Nuclear magnetic resonance (NMR) spectra were taken on a Varian 220 MHz instrument and on a Varian A-60. Thanks is given to Dr. Melvin Klein for assistance in operating these instruments. Gas liquid chromatography (GLC) was done on a Varian Aerograph 204 instrument equipped with a thermal conductivity detector. When a flame ionization instrument was needed, a F&M 5750 was used. Mass spectra were taken on AEI MS 12 by Jerry Han. Scintillation counting was done on a Packard Model 3375 liquid scintillation counter. The scintillation fluid consisted of 4.5 grams 2,5-diphenyl oxazole and 0.5 grams p-bis-2-(5-phenyl oxazole)-benzene per liter of toluene. If aqueous solutions were counted, Beckman "Bio-Solv" solubilizers were added along with the aqueous solution to the scintillation fluid. Autoradiography was done with a Geiger tube built in this laboratory.

Introduction

As with any exploratory scientific inquiry, the original questions asked lead to many different questions.

This is the evolution of my thesis problem. I started looking at the photochemical behavior of aromatic N-oxides in solution. There was a report that oxygen could be produced by the gas phase photolysis of pyridine N-oxide (Hata and Tanaka, 1962) (Fig. 1). This was a curious result and brought up speculations concerning the nature of the nitrogen-oxygen bond in such chemical systems. There had been some work done on similar structures in aliphatic compounds. Such a structure, (I), was found by J. Splitter and M. Calvin (1965) to produce an oxaziridine, (II), under photolytic conditions:

These oxaziridines, described chemically by Emmons (1957), proved to be good oxidants. This brought up the possibility that the primary product in the photolysis of pyridine N-oxide was such an oxaziridine which then produced oxygen, possibly by a bimolecular mechanism. So I started by looking at the solution photochemistry of pyridine N-oxide, since it was the simplest of the aromatic N-oxides.

The photochemistry of pyridine N-oxide turned out to be quite

HATA & TANAKA (1962)

STREITH et al. (1967)

XBL 684-4186 A

Figure 1. Photochemical reactions of pyridine N-oxide.

complicated. By analyzing the products, I found that the solvent was being oxidized during the photochemical reaction--i.e., acetaldehyde was produced from ethanol. This oxidation was very intriguing, bringing up the question of where the oxygen was going since pyridine was also a product of the photolysis. A very interesting question arose: Could the oxygen be transferred to another molecule? At this time a paper came out by Chaykin (1966) describing oxygen transfer from nicotinamide N-oxide to xanthine catalyzed by xanthine oxidase. With this information, the problem became one of more general nature than exploring interesting chemistry. The possibility became apparent that these aromatic N-oxides could act as oxygen sources in biological systems. Thus I embarked on the biochemical aspects of these aromatic N-oxides.

At the same time, in investigating the photochemical oxygen transfer, the substrate I chose was cyclohexene. I hoped to produce the epoxide by transfer of oxygen from pyridine N-oxide. At first the experiment failed due to difficulties in the experimental procedures. Then Streith and Sigwald (1967) (Fig. 1) published a paper describing the photochemical transfer of oxygen from pyridine N-oxide to benzene to produce phenol. With renewed efforts, I was able to find the correct conditions to produce the cyclohexene oxide from cyclohexene and pyridine N-oxide.

With this reaction established, the biological aspects were pursued. These experiments and the results are reported in the second part of the thesis.

Since my experiments, several key papers appeared substantiating the oxygen transfer reaction and relating this reaction to possible biological systems. In 1969, T. Tsuchiya et al. reported the production of cyclohexene oxide from pyridazine N-oxides and cyclohexene, using the same photolytic conditions that I used. Also, very soon afterwards, there appeared a paper by Jerina, Boyd et al. (1970) showing that pyridine N-oxide transferred its oxygen to a variety of aromatic and aliphatic substrates. The oxygen transfer paralleled the action of hydroxylase enzyme systems. Most exciting was their discovery that this photochemical model system reproduced the enzymatic "NIH shift". Thus the photochemical transfer of oxygen seems to be the best model system for studying the "activated" oxygen which hydroxylates substrates catalyzed by the oxygenase enzymes.

In this first section, I would like to describe the photochemical experiments which lead up to the development of such a model system for hydroxylases.

I-2 THE PHOTOCHEMISTRY OF PYRIDINE N-OXIDE

The Photochemistry of Pyridine N-oxide

Introduction

The main thrust of the experiments presented in this and the subsequent sections is to develop the photolytic reactions of aromatic N-oxides as a model for biological oxidations. In particular, the photochemical transfer of oxygen from pyridine N-oxide to a substrate may provide a key to the intermediate in oxygen activation by enzymes.

In this section, the products of the photolysis of pyridine N-oxide will be described. These results have been published (A. Alkaitis and M. Calvin, 1967). There is an excellent review which is quite extensive and complete which includes the photochemistry of various aromatic N-oxides (G. Spence, E. Taylor, O. Buchardt, 1970). This review puts these results which I will report into perspective regarding the chemical and photochemical behavior of aromatic N-oxides. The chemistry of amine oxides is very well reviewed in Ochiai's book (1967).

Experimental

The synthesis of pyridine N-oxide and other compounds described in this section will be discussed in the synthetic section. Acetaldehyde was purchased from Aldrich Chemical Company. Spectroquality pyridine was purchased from Matheson, Coleman and Bell. GLC column packing materials were obtained from Applied Sciences Lab.

The photolysis were carried out in a quartz water cooled immersion apparatus equipped with a Hanovia 450W high pressure mercury lamp. The emitted light was filtered by means of various glass

sleeves which surrounded the light. The solutions were stirred by means of a magnetic stirrer. Nitrogen was bubbled through the solution through a sintered glass tube built into the solution chamber.

The pyridine N-oxide was distilled, then purified by sublimation. The sublimator was opened in a dry box and the pyridine N-oxide was transferred immediately into a volumetric flask, weighed, and dry solvent added. The above manipulation was needed since pyridine N-oxide is extremely hygroscopic. A typical photolysis solution consisted of approximately 1 gram pyridine N-oxide/100 ml solvent.

At the end of the photolysis, the reaction mixture was analyzed by gas-liquid chromatography (GLC). A stainless steel column (6 ft. x 1/8 inch), packed with a 2.5% FFAP on Chrom G column material, was used extensively. A Porapak Q (3 ft. x 1/8 inch) column was used to separate acetaldehyde from ethanol. In order to identify the products and assign the GLC peaks properly, the volume of the photolysis mixture was reduced in vacuo and the resulting concentrated solution was separated by means of a preparative GLC. An 8 ft. x 1/4 inch column was used for this purpose. A 10:1 effluent splitter was used with one part of the effluent gas going to the flame detector and the other 9 parts going to a collection port.

The quantities of products formed were determined by measuring areas under the peaks and comparing these to areas from standard solutions of the compounds.

Results

Fig. 1 shows the results of a typical run (photolysis of 1 g/100 ml RCH_2OH , 10 mole). With the pyrex filter, 50% of the starting material was recovered after 6 hours of photolysis. Using an all quartz system, 10% of the starting material was recovered after 10 min of photolysis.

Structure Determination of Products

The structure of I, II, IV, and V was proved by comparison of the GLC retention times, the IR spectra, the UV spectra, the NMR spectra, and the mass spectra to those of authentic samples (I and II were available commercially; IV and V were synthesized).

The structure of III is believed to be as shown from the following evidence: the IR spectrum shows no N-H stretch at 3400 cm^{-1} and no carbonyl stretch. there is a strong absorption pattern at 1000 to 1550 cm^{-1} indicative of an acetal function. The NMR spectrum of III ($\text{R}=\text{H}$) shows a sharp singlet at $3.2\ \delta$ (6 H) and broad singlets at $5.7\ \delta$ (1 H), $6.05\ \delta$ (2 H), $6.75\ \delta$ (2 H). The NMR spectrum of III ($\text{R}=\text{CH}_3$) shows a triplet at $1.2\ \delta$ (6 H), a quarter at $3.5\ \delta$ (4 H), and broad singlets at $5.9\ \delta$ (1 H), $6.08\ \delta$ (2 H), $6.8\ \delta$ (2 H).

The UV spectrum in EtOH of both $\text{R}=\text{H}$ and $\text{R}=\text{CH}_3$ shows a λ_{max} at $220\text{ m}\mu$, ϵ 5,600. Upon addition of one drop of 0.1 N HCl the λ_{max} shifted to $235\text{ m}\mu$ with an increase in ϵ . Upon addition of 3 drops of 0.1 N NaOH the λ_{max} shifted to $210\text{ m}\mu$. Upon reacidification, there is no further shift in the λ_{max} . N-formyl pyrrole (synthesized as a standard for proof of V) shows a λ_{max} in EtOH at $235\text{ m}\mu$, ϵ 11,200,

	I	II	III	IV	V
10 mmole					
Pyrex filter	0.26 mmole	0.68 mmole	0.27 mmole	0.13 mmole	trace
All quartz	0.42 mmole	0.69 mmole	0.36 mmole	0.50 mmole	trace

XBL 684-4185

Figure 1. Photolysis of pyridine N-oxide in ethanol and methanol.

(See text for experimental details.)

in the UV. Upon addition of 2 drops of 0.1 N NaOH, there is a shift of the $\lambda_{\max}$ to 210 m μ . A parallel experiment was performed on a more concentrated solution of III and the reaction with acid or base was followed by GLC. The product of each of the reactions was collected and the UV taken of the fractions. The following reaction is consistent with the results. (It was found that III slowly hydrolyzed to N-formyl pyrrole upon standing in ethanol solution.)

Elemental analysis of III, R=CH₃, agreed with the formula C₉H₁₅NO₂. A high resolution mass spectrum was taken of III (R=H). (Thanks to Dr. H. Schnoess.) The base peak was m/e 75 (C₃H₇O₂). The other major peaks were: parent peak (18% of base peak) at 141 (C₇H₁₁NO₂); m-31, C₆H₈NO (32% base); m-46, C₅H₅NO (30% base); m-74, C₄H₅N (40% base). The following scheme gives the formulation to the fragmentation pattern.

Discussion

The photochemistry of the aromatic N-oxides is interpreted in the light of the photochemistry of the nitrones since both have the same atomic arrangement around the nitrogen atom. The primary product of nitrone irradiation is the oxaziridine. Figure 2 gives the chemical formation of the oxaziridine and the identification of the oxaziridine structure (from H. Ono's thesis, 1969). Figure 3 shows some of the general reactions of the oxaziridines. Note the oxidation of iodide and the thermal rearrangement to the amide.

In the photochemistry of the aromatic N-oxides, the most extensively studied series was the quinoline N-oxides. Figure 4 shows the primary rearranged photoproducts. The parent quinoline compounds

- I R = NO₂; R' C₂H₅
 II R = NO₂; R' t-C₄H₉
 III R = H; R' t-C₄H₉

Identification by:

UV, IR, active O

Mix. mp., UV, active O

UV, active O, isom. to Nitron.

Splitter and Calvin (1958)

Emmons (1957)

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Figure 2. Photochemical and chemical formation of the oxaziridine structure (from H. Ono, Ph.D. Thesis, University of California, 1969).

GENERAL REACTIONS OF OXAZIRIDINES

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Figure 3. General reactions of oxaziridines: solvolysis, rearrangement, and reduction of the oxaziridine.

BUCHARDT (1966)

KANEKO (1966)

XBL 684-4190

Figure 4. Primary products of the photolysis of substituted quinoline N-oxide.

were also formed in varying yields. A discussion of these and minor sideproducts is given completely in the review cited (G. Spence *et al.*, 1970). Although the pyridone derivatives were reported as products of the photolysis of quinoline N-oxides, no pyridone could be detected in the pyridine N-oxide photolysis. Figure 5 shows some of the other products isolated as coming from secondary reaction of the primary formed 1,3-benzoxazepine. These products were only identified by means of an NMR. Very likely, III comes from such an intermediate oxazepine. However, the oxazepine from pyridine N-oxide must be completely unstable, since there is no spectral evidence for such an intermediate in all the photolysis experiments with pyridine N-oxide. (The UV of the oxazepines has an absorption band around 340 nm. Pyridine N-oxide, upon irradiation, showed no shift in the UV absorption band to longer wavelengths.) The photochemistry of pyridine N-oxide did correspond to that of the quinoline N-oxides.

It is generally postulated by analogy to the nitron photochemistry (Fig. 2) that the first step in the photochemical reaction of the aromatic N-oxide is the formation of the non-isolatable, unstable oxaziridine, with subsequent rearrangement.

XBL 684-4192

Figure 5. Reactions of benz-1,3 oxazepine with alcoholic solvents (Kabeko et al. (1966)).

The finding of the previously unreported oxidation product of the solvent (acetaldehyde from ethanol) is evidence for the more powerful oxidant, an oxaziridine, as an intermediate. It is known that an oxaziridine will oxidize iodide to iodine. Oxazepines, on the contrary, will reduce iodine to iodide (O. Buchardt, 1966). Since, in the oxidation of iodide by an oxaziridine the parent imine is formed as a product, the presence of the deoxygenated heteroatomic compound may also be explained by the following reaction:

VI

If the solvent is susceptible to oxidation by some photochemical intermediate, it may be possible to oxidize a carbon-carbon double bond and obtain an oxygen transfer (perhaps to an epoxide) instead of a hydrogen abstraction as in the case of ethanol. Photochemical transfer of oxygen from pyridine N-oxide to cyclohexene is discussed in Section 4.

Synthesis of Pyridine N-oxide

The method described by E. Ochiai (1953) was employed. To a solution of 40 g pyridine in 300 ml glacial acetic acid, 50 ml of

35% aqueous hydrogen peroxide was added. The mixture was heated on a water bath at 80°C. After 3 hours an additional 35 ml peroxide (total of 1.7 moles) was added and the mixture was maintained at 80°C for an additional 9 hours. The mixture was concentrated to approximately 100 ml in vacuum, diluted with 100 ml water and again concentrated in vacuum as far as possible. The residue was made strongly alkaline with anhydrous sodium carbonate, shaken with 250 ml chloroform and allowed to stand. The solution was filtered, dried with sodium sulfate, and the solvent removed. The residue was vacuum distilled. The yield of pyridine N-oxide was 46 g (96% theoretical). The boiling point at 15 mm mercury was 138-140°C. The melting point, taken in a sealed tube, was 66-67.5°C. The main IR absorption bands in cm^{-1} are: 3300, 2950, 1700, 1450, 1250, 1185, 1160, 1015, 900, 840. The UV absorption maxima in methanol (molar extinction coefficients in parenthesis) are at 2630 Å (12,800) and 2110 Å (17,800).

Synthesis of Pyrrole Aldehyde (Pyrrole-2-carboxaldehyde)

The method used was the same as the one described in Organic Synthesis Coll. Vol. IV, 831 (1963). The UV absorption maxima in alcohol were 278 nm ($\epsilon = 16,500$) and 238 nm ($\epsilon = 6,500$). The IR maxima are in cm^{-1} : 3400, 3200, 2750, 1645, 1400, 1340, 1300, 1280, 1110, 1070, 1060, 1020. The NMR (on Varian A-60) showed multiplets centered at δ 6.2, 6.8, 7.1 and doublet centered at δ 9.3 ($J = 2$ cps). Each of these absorption bands integrated for 1 proton. (The N-H proton is very broad at δ 10.) The mass spectra (relative intensities in parenthesis) showed m/e at: 96(8), 95(100), 94(63), 93(2),

68(3), 67(39), 66(42), 65(1), 42(5), 41(5), 40(13), 39(6), 38(4). The mass spectra was taken on an AEI MS12.

Synthesis of N-Formyl Pyrrole

N-formyl pyrrole was able to be synthesized by a modification of the procedure used by N. Putochin (1926). (If the precise procedure is employed as described by Putochin, only pyrrole-2-aldehyde is obtained.) The synthetic route is outlined in the following reaction sequence:

To a 50 ml 3-neck round bottom flask, fitted with a dropping funnel, condensor, and mechanical stirrer with teflon paddle, is added first 2 ml ether and 5.6 g magnesium and then is added in a 15-min interval a solution consisting of 30 g ethyl iodide in 30 ml ether. The solution is then allowed to stir for an additional hour. To the grey solution is added 13 g of fresh distilled pyrrole in 8 ml ether. The rate of addition of the pyrrole is adjusted to the

rate of formation of gas (bubbles in the solution). Vigorous bubbling subsided after half of the pyrrole had been added. The grey-green solution is cooled to -10°C by dry ice-acetone bath. To the cool reaction vessel is added very slowly 40 g of ethyl formate. It is extremely important to add the formate slowly enough and to keep the flask in a cold bath so that the temperature in the reaction vessel does not exceed approximately -10°C . The grey-green solution turns to a reddish-green syrup as the formate is being added. Additional ether was added to keep the viscous mass from solidifying. After the addition of the formate, 30 ml cold water is added. The dark brown mixture is extracted with ether. The ether extracts are distilled under reduced pressure. The fraction boiling over at $40-50^{\circ}\text{C}$ (22 mm Hg) contained the product. This distilled fraction was then purified by means of preparative GLC (25 ft x 1/4 inch column, 5% SE 30 on Chrom W). The yield of N-formyl pyrrole was approximately 10% of the theoretical yield.

The UV absorption maxima was at 235 nm ($\epsilon = 10,000$). The IR spectrum showed the following maxima (cm^{-1}): 2800, 1730, 1720, 1520, 1460, 1390, 1320, 1280, 1230, 1060, 1040, 930. The NMR (on Varian A-60) spectra showed broad singlet absorption bands at 6.3 (2 protons), 7.3 (2 protons), and 8.8 (1 proton) ppm.

The fragmentation pattern from the mass spectrum is shown below:

<u>m/e</u>	<u>Relative intensity</u>	<u>m/e</u>	<u>Relative intensity</u>
37	21	62	1
38	40	63	3
39	100	64	3
40	71	65	2
41	87	66	12
42	4	67	52
44	14	68	4
48	1	94	2
49	3	95	80
50	8	96	7
51	6		
52	5		

Chemical behavior of N-formyl pyrrole

It was found that N-formyl pyrrole was insensitive to mild acid. There was no change in the UV absorption bands or in the retention times on the GLC upon addition of a few drops of concentrated HCl to a methanol solution of N-formyl pyrrole. Upon the addition of a few drops of 1 M NaOH solution, the UV absorption maximum shifted to 210 nm, and the retention times of the product on the GLC corresponded to pyrrole. This behavior is consistent with the behavior of the N-formyl pyrrole produced by the hydrolysis of the product (III) from irradiation of pyridine N-oxide in alcohol.

IR and Mass Spectra of III

A. R=H

The IR spectra showed the following absorption maxima in cm^{-1} :
3000, 2920, 2870, 2760, 1700, 1470, 1430, 1370, 1310, 1270, 1240,
1200, 1180, 1170, 1100, 1060, 1050, 980, 950, 890.

The fragmentation pattern of a high resolution mass spectrum taken on a Consolidated Electronics Model 110 mass spectrometer (70 eV, source temp 270°C) is given below:

<u>m/e</u>	<u>Formula</u>	<u>Relative intensity</u>
	C H N O	
38	3 2 0 0	13
39	3 3 0 0	49
40	3 4 0 0	18
41	2 3 1 0	32
42	2 4 1 0	2
50	4 2 0 0	4
51	4 3 0 0	7
52	4 4 0 0	4
53	4 5 0 0	4
66	4 4 1 0	8
67	4 5 1 0	40
75	3 7 0 2	100
76	3 8 0 2	5
80	5 6 1 0	7
81	5 7 1 0	6

(Continued)

<u>m/e</u>	<u>Formula</u>	<u>Relative intensity</u>
94	5 4 1 1	4
95	5 5 1 1	28
110	6 8 1 1	30
141	7 11 1 2	17

B. $R=CH_3$

The IR spectra showed the following absorption maxima in cm^{-1} :
3000, 2920, 2870, 2850, 1540, 1500, 1470, 1430, 1410, 1370, 1310,
1270, 1240, 1100, 1060, 1050, 950, 900, 830.

The fragmentation pattern of a low resolution mass spectrum taken on an AEI Model MS 12 mass spectrometer (70 eV, source temp 200°C, glass inlet) is given below:

<u>m/e</u>	<u>Relative intensity</u>	<u>m/e</u>	<u>Relative intensity</u>
37	3	67	35
38	6	68	31
39	22	69	2
40	15	75	44
41	28	76	2
42	4	79	1
43	9	80	8
44	19	81	1
45	9	94	3
46	2	95	11
47	100	96	28
48	2	97	2
49	1	103	65
50	2	104	6
51	3	124	21
52	3	125	2
53	2	169	6
66	6	170	0.6

I-3 THE PHOTOCHEMISTRY OF NICOTINAMIDE N-OXIDE

The Photochemistry of Nicotinamide N-Oxide

Introduction

Since nicotinamide is the aromatic heterocyclic compound implicated in biological oxidation reactions (see Section II-1), the photochemistry of nicotinamide N-oxide was studied. The problem with the study of the photochemical properties of nicotinamide N-oxide is that the material is not soluble in many solvents. Ten grams nicotinamide N-oxide dissolve in 1 ml water. It is slightly soluble in methanol and ethanol (about 0.1 g/ml) and essentially insoluble in all the other common organic solvents. Thus it was impossible to test whether nicotinamide N-oxide transferred oxygen to an olefin under photolytic conditions. It was, however, photoreactive in water, ethanol, or methanol. This was determined by the changes in the UV absorption spectrum: the 268 nm and 222 nm peaks decreased and a new absorption peak at 355 nm appeared. Although this particular photochemistry does not directly relate to the model system for biological hydroxylations, it adds to the elucidation of the photochemical behavior of derivatives of pyridine N-oxide. There is certainly a marked change in the photochemical behavior of a pyridine N-oxide when a substituent group is placed in the 3 position.

Experimental

Nicotinamide N-oxide was synthesized using the procedure of E. Taylor and A. Croveti (1954). The nicotinamide N-oxide was recrystallized several times from a water-ethanol mixture. The

characteristics of the product were the same as those reported for nicotinamide N-oxide in the literature. The m.p. was 290-292°C (dec.). The UV spectrum showed absorption maxima at 268 nm ($\epsilon = 11,000$) and at 222 ($\epsilon = 20,000$). The mass spectrum showed a large parent peak (30% of the base peak) at m/e 138 and a large peak (base peak) at m/e 122.

Nicotinamide N-oxide was first irradiated using the same experimental procedure used in the irradiation of pyridine N-oxide. Approximately 50 mg/100 ml solvent (methanol, ethanol, water) were irradiated with a high pressure 450W Hanovia mercury lamp. The same results, with only a difference in the time course, were obtained from the irradiation of the nicotinamide N-oxide with or without a pyrex filter. The reaction was monitored by taking a UV absorption spectra of aliquots taken at various times. It was found that initially only one soluble product was formed with an absorption peak at 355 nm. Under these conditions (quartz reactor) the initially formed product reached its height after 4 min of irradiation and was subsequently photolyzed. The final photolysis product was not identified. The fact that one soluble product was initially formed is deduced from the fact that there was an initial linear decrease in the UV absorption of nicotinamide at 268 nm with a concomitant linear increase in the absorption at 355 nm. (An insoluble polymer also seems to form with irradiation.) There was an isoaspectric point at 310 nm. However, the initially formed product, although it was stable in solution, decomposed or rearranged to several unidentified

products when subjected to chromatographic procedures. Differential extraction with various solvents and attempts to crystallize the product failed.

Therefore, the initially formed product from the irradiation of nicotinamide N-oxide in D_2O was studied by means of NMR spectroscopy and UV spectroscopy.

A solution of 5 mg nicotinamide N-oxide in 1 ml D_2O was placed in an NMR tube. The NMR spectrum was recorded on a 220 MHz spectrophotometer (Varian Associates). The NMR tube was then placed in a rayonette photolysis apparatus equipped with 2537 Å lamps. After set times (81, 360, and 577 min) of irradiation, the UV absorption spectrum and the NMR spectrum were recorded. The UV spectrum was determined by removing 15 λ of the irradiated solution, diluting it with 15 ml H_2O , and recording the UV absorption spectrum.

Results

The UV absorption spectrum, taken at the indicated times of irradiation, of nicotinamide N-oxide in D_2O is shown in Figure 1. The 350 nm absorption peak increased with time of irradiation, whereas the 265 nm and the 222 nm peaks decreased. Despite the length of time that the sample was irradiated, a single soluble product seemed to be formed as evidenced by the simple UV absorption spectra and a very clean NMR spectra (one set of absorption peaks disappear and another set of absorption peaks appear). However, the intensity of the NMR peaks diminished with increased times of irradiation. This is indicative that some insoluble side product, or subsequent

XBL 703-5121

Figure 1. The UV absorption spectra of a solution of nicotinamide N-oxide in D₂O, after irradiation for various lengths of time. See text for details.

IRRADIATION OF NICOTINAMIDE N-OXIDE
(5 mg / 1 ml D₂O in NMR tube)

220 MHz NMR

$\nu_a = 7.75$	$J_{ab} = 8.0$	$\nu_a = 7.63$	$J_{ab} = 8.0$
$\nu_b = 8.11$	$J_{ac} = 6.0$	$\nu_b = 8.26$	$J_{ac} = 4.5$
$\nu_c = 8.46$	$J_{ad} = 0.3$	$\nu_c = 8.70$	$J_{ad} = 1.2$
$\nu_d = 8.72$	$J_{bc} = 1.8$	$\nu_d = 8.92$	$J_{bc} = 3.0$
	$J_{bd} = 0.5$		$J_{bd} = 1.8$
	$J_{cd} = 1.0$		$J_{cd} = 1.2$

XBL 703-5120

Figure 2. Assigned structure of the product of irradiation of nicotinamide N-oxide in D₂O. Whether the product is a 1,2 or a 1,3 oxazepine is unable to be determined. The NMR (220 MHz, Varian Associates instrument) of the starting nicotinamide N-oxide and the product of the irradiation is given. The chemical shifts are in delta units. J is the coupling constant in cps.

insoluble rearranged product, is being formed. Figure 2 shows the NMR spectrum of the starting nicotinamide N-oxide and of the resultant product after irradiation for 577 min. The possible structures of the photolysis product which are consistent with the data are drawn. This assignment is tentative until the product is isolated and purified.

The NMR spectra, taken at the times of irradiation indicated, showed a very clean conversion to a single soluble product. The nicotinamide N-oxide had absorption peaks in the NMR at 7.75, 8.11, 8.46, and 8.72 ppm. Each peak integrated to one proton. After 81 min of irradiation, three of the NMR peaks decreased in height (7.75, 8.11, 8.46 ppm) and three new peaks appeared (7.63, 8.26, 8.92 ppm). After 360 min of irradiation, it appeared that approximately half of the nicotinamide N-oxide had been converted to the product. The NMR spectrum showed 7 absorption bands integrating in a ratio of 1:2:1:1:1:1:1 at 8.92, 8.70, 8.46, 8.26, 8.11, 7.75, and 7.63 ppm. After 577 min of irradiation, the NMR peaks of the original nicotinamide N-oxide had disappeared, and only four peaks remained at 7.63, 8.26, 8.70, and 8.92. These peaks integrated with a ratio of 1:1:1:1.

Discussion

The product of the photolysis of nicotinamide N-oxide is very different from the products of the photolysis of pyridine N-oxide. The formation of a 7-member ring when nicotinamide N-oxide is irradiated may indicate that the same type of 7-member ring is formed on irradiation of pyridine N-oxide. This 7-member ring, formed from pyridine N-oxide, would not be stabilized by a carboxamide group and would

rearrange to give the final isolable products as reported in the previous section. Also, the formation of such a 7-member ring as an intermediate in the photolysis of aromatic N-oxides (benzoxazepines have been isolated from the photolysis of quinoline N-oxides) lends support to an initially formed oxaziridine structure. The oxaziridine-oxazepine rearrangement is analogous to the benzene oxide-oxepine rearrangement.

The assignment of the oxazepine structure as the soluble product of the irradiation of nicotinamide N-oxide is consistent with the spectral data presented. The oxazepine structure has been unequivocally identified (by X-ray crystallography) as a product in the photolysis of quinoline N-oxides (Buchardt et al., 1970). It is not possible to determine whether the product from irradiation of nicotinamide N-oxide is the 1,2 or the 1,3 oxazepine. The 1,3 oxazepine is the one formed on irradiation of quinoline N-oxides. It would be extremely interesting if the 1,2 oxazepine were the structure formed on irradiation of nicotinamide N-oxide. This would be the first 1,2 oxazepine found. A study of the substituent effects on the photolysis of the pyridine N-oxide could provide insights into the mechanism of the photolytic reaction.

I-4 PHOTOCHEMICAL OXYGEN TRANSFER FROM PYRIDINE N-OXIDE TO CYCLOHEXENE

Photochemical Oxygen Transfer from Pyridine N-oxide to Cyclohexene

Introduction

It was found (Section I-2) that pyridine N-oxide, upon irradiation with ultraviolet light, can be converted to an active intermediate capable of oxidizing an alcohol to an aldehyde. Two questions immediately arise: What is the nature of this active intermediate, and can this active intermediate transfer oxygen to some acceptor molecule? The nature of this active intermediate will be discussed in the next section. In this section the transfer of oxygen under photolytic conditions from pyridine N-oxide to cyclohexene will be presented. Cyclohexene was chosen as an oxygen acceptor since all of the simple oxygenated products of cyclohexene were readily available.

Experimental

Cyclohexene, chromatography, was purchased from Matheson, Coleman, and Bell. It was found by GLC that this cyclohexene contained appreciable quantities of oxygenated products, particularly cyclohexenol and cyclohexenone. (Purified cyclohexene upon standing in air will rapidly form cyclohexenone and cyclohexenol.) Therefore the cyclohexene was purified by GLC and stored under helium before using. The GLC instrument used was a F&M Model 775 equipped with a 12 ft x 3/4 inch column (20% Carbowax 20M on Chrom W, Applied Science). Pyridine N-oxide was used in the same manner as described in the previous section (I-2). Cyclohexene oxide, cyclohexanol, and cyclohexanone were purchased from Aldrich Chemical Co. Cyclohexenone and cyclohexenol were a gift of Mr. Bruck Radcliff in the Chemistry Department.

Pyridine N-oxide was not soluble in the cyclohexene to any appreciable amount. It was found that pyridine N-oxide was very soluble in methylene chloride, and was photoreactive in this solvent. Therefore pyridine N-oxide was made soluble in cyclohexene by addition of 20% methylene chloride. The irradiation (0.5 g pyridine N-oxide in 20% methylene chloride in cyclohexene) was carried out in a quartz, water-cooled immersion apparatus equipped with a Hanovia 450W high pressure mercury lamp. A vycor sleeve was used to filter the light. The photolysis solution was flushed with dry nitrogen (scrubbed with basic pyrogallol) for 45 min before the irradiation was started. After the irradiation was complete, samples of the photolysis mixture were injected into a glc (F&M Model 5750) equipped with a 2.5% FFAP column (on Gaschrom Q, Applied Sciences). The quantities of products formed were determined by measuring the area under each peak. Areas were related to quantity by measuring areas under peaks of known concentration of standard compounds.

Results

The results of a typical run are given in Figure 1. The figure shows a glc trace of the products obtained from an 8-min photolysis of pyridine N-oxide in cyclohexene (20% methylene chloride). Approximately 1/2 of the pyridine N-oxide remained after 8 min of photolysis. The quantities of the products formed are given below. (Initial amount of pyridine N-oxide = 5 mmoles.)

<u>Product</u>	<u>mmoles formed</u>
cyclohexene oxide	0.1
pyridine	0.8
cyclohexanone	0.1
cyclohexanol	0.01
2 formyl Pyrrole	0.1

XBL 685-4209

Figure 1.

The structures of the products were assigned by collecting the products by preparative glc and comparing the glc retention times by coinjection and their mass spectra with standards. The fragmentation patterns obtained from the mass spectra of the various photolysis products matched perfectly with the fragmentation patterns of the assigned standards. The major peaks from the fragmentation patterns of these products are given in Table I.

Discussion

Cyclohexenol and cyclohexenone do not seem to be products of oxygen transfer from pyridine N-oxide to cyclohexene. In the purification of cyclohexene there remains a small quantity of the peak identified as cyclohexenone and a small peak identified as cyclohexenol. Upon irradiation of pyridine N-oxide in this purified cyclohexene, the peak corresponding to cyclohexenone does not change in height, whereas the peak corresponding to cyclohexenol does raise in height (to about twice its original height). This rise could come by the interaction of the cyclohexene with air. In the purified cyclohexene, if allowed to stand in air (also if the photolysis mixture is allowed to stand in air), cyclohexenol and cyclohexenone will form rapidly. A control photolysis, using only purified cyclohexene (no N-oxide) under the same conditions which gave oxygen transfer from pyridine N-oxide, showed that no oxygenated products formed from the cyclohexene by itself.

In another control experiment using the usual photolysis conditions, the disappearance of pyridine N-oxide was followed with time and the appearance of cyclohexene oxide was followed with time. The

Table I

Mass Spectra of Products of Photolysis of Pyridine N-oxide in Cyclohexene

Cyclohexene oxide		Pyridine	
<u>m/e</u>	<u>Relative intensity</u>	<u>m/e</u>	<u>Relative intensity</u>
39	34	50	10
40	7	51	18
41	82	52	44
42	58	53	6
43	20	78	11
44	82	79	100
55	48	80	7
56	19		
57	67	Cyclohexenol	
67	15	<u>m/e</u>	<u>Relative intensity</u>
68	46	39	9
69	75	40	7
70	42	41	16
79	14	42	7
80	23	43	9
83	100	44	24
97	23	54	11
98	32	55	11
		56	15
Cyclohexanone		57	100
<u>m/e</u>	<u>Relative intensity</u>	58	8
41	20	67	20
42	52	71	15
43	5	72	8
44	14	77	11
54	7	78	6
55	100	79	45
56	7	80	23
69	34	81	7
70	26	82	60
80	8	83	6
83	9	98	3
98	68	99	2
99	4	100	4

pyridine N-oxide disappearance and the cyclohexene appearance was linear with time for the first 6 min of irradiation.

The material balance is not good. All of the pyridine N-oxide consumed cannot be accounted for by the products isolated. There is considerable polymer formed, which could explain where the rest of the pyridine N-oxide went. The material balance for the oxygen transfer reaction is also not good. There is 0.8 mmole of pyridine formed and only 0.2 mmole of oxygenated cyclohexene formed. It is possible that some of the oxygen lost from the pyridine N-oxide could oxygenate the polymer formed (possibly the oxygen could be intimately connected in the production of the polymer). Another possibility is that molecular oxygen is formed in solution, paralleling the finding of Hata and Tanaka (1962) that molecular oxygen is formed in the gas phase photolysis of pyridine N-oxide. This possibility could be checked with difficulty by photolyzing pyridine N-oxide O^{18} . The fact that the material balance is not good can be due to polymer formation in the concentrated pyridine N-oxide solution. When pyridine N-oxide was photolyzed in cyclohexene in a more dilute solution (0.3 g/100 ml), and with a pyrex filter, the material balance was better. After approximately 2/3 of the pyridine N-oxide was photolyzed (approximately 2 mmoles), 0.4 mmole cyclohexene oxide, 1.1 mmoles pyridine, and 0.15 mmole cyclohexenone were formed.

Conclusion

The photochemical transfer of oxygen from pyridine N-oxide to cyclohexene has been shown. This finding has since been substantiated

by T. Tsuchiya et al. (1969). The photochemical transfer of oxygen to a substrate from pyridine N-oxide seems to be a general reaction. There is some active intermediate formed, possibly atomic oxygen or the oxaziridine, which acts as a good oxidant. There is some extremely interesting chemistry involving the nature of the nitrogen-oxygen bond which is worthy of study. The particular aspect of this oxygen transfer reaction which interested me was the possibility that this reaction could serve as a model system for the activation of oxygen by oxygenase enzymes. This direction of inquiry will be discussed in the following section.

I-5 PHOTOLYTIC OXYGEN TRANSFER AS A MODEL FOR MICROSOMAL HYDROXYLASE

Photolytic Oxygen Transfer as a Model for Microsomal Hydroxylase

With the discovery that pyridine N-oxide, upon irradiation, transfers its oxygen to a variety of substrates, the possibility arises that this photochemical transfer reaction may serve as a model system for the activation of molecular oxygen by mixed function oxidases. The number of substrates oxygenated and the products formed from photolytic oxygen transfer parallel the hydroxylation of various substrates by mixed-function oxidase systems.

The mixed-function oxidase system hydroxylates a wide variety of substrates: olefins to epoxides, aromatics to phenols, and aliphatics to carbinols. Several important aspects of the hydroxylase system have been elucidated: 1) The reaction is stereospecific. R. McMahon and H. Sullivan (1969) found that on hydroxylation of ethyl benzene by liver microsomes, 86% of the methyl phenyl carbinol formed was R(+). 2) In the hydroxylation of aliphatics by microorganisms there is a direct substitution of a hydrogen by a hydroxyl group. The reaction is stereospecific and proceeds with a net retention of configuration (E. Heinz et al., 1969; and D. Jones, 1968). 3) In para hydroxylation by microsomes of aromatic compounds substituted in the para position (T, CH₃, Br, for example), the p-hydroxy aromatic compound retains the original para substituent. This is called the NIH shift. The figure below (S. Udenfriend et al., 1969) gives an example of the NIH shift:

These above observations on the action of microsomal hydroxylase systems, coupled with studies on model systems, provide some clues to the nature of the hydroxylating species which must be formed from molecular oxygen.

There are several chemical systems which will hydroxylate various substrates. These chemical hydroxylation systems can serve as models in the elucidation of the mechanism of microsomal hydroxylase systems. The major question which can be investigated by using model systems concerns the chemical nature of the hydroxylating species derived from molecular oxygen. The comparison of these various model systems and microsomal hydroxylase systems is presented by V. Ullrich and H. Staudinger (1969). Previous to the discovery of the photochemical model system, most of these model hydroxylase systems contained a reduced metal and some form of oxygen (either molecular oxygen or hydrogen peroxide). Comparing the action of these various chemical

hydroxylating systems on different substrates, the nature of the hydroxylating species (whether it is an OH radical, and OH^+ , or an "oxene") can be deduced. Then, comparing the hydroxylation produced by microsome to the various model hydroxylations, it can be concluded that the active oxygen species produced by microsomal hydroxylase systems is an electrophilic oxygen (possibly OH^+) or an oxene. An oxene is an oxygen nucleus with six electrons around it (analogous to the carbene).

The main reason for invoking an OH^+ as the hydroxylating species has been the observation of the NIH shift. Recently, with the discovery that arene epoxides are metabolites of aromatic compounds (D. Jerina et al., 1968), the NIH shift may be explained by an initial formation of an epoxide intermediate (D. Jerina, J. Daly, et al., 1970). This epoxide intermediate could possibly be formed by an "oxene" reacting with the aromatic compound, thus eliminating the need for an OH^+ oxygen. None of the model hydroxylating systems were able to reproduce the NIH shift. Hydroxylation by peroxytrifluoroacetic acid did reproduce the NIH shift to a slight degree (D. Jerina, 1969). However, the NIH shift produced by this model system did not correspond to the NIH shift produced by microsomes when tested with various substrates.

The discovery that the photochemical oxygen transfer could take place made possible another model system. Jerina, Boyd, and Daly (1970) looked at the photochemical transfer of oxygen from pyridine N-oxide to various substrates. These results were compared to

microsomal hydroxylation of these same substrates. He found that the photochemical oxygen transfer reaction was the best model system to date. It reproduced the NIH shift to a similar degree that the micorsomes produced the NIH shift in all substrates tried. 1,2-naphthalene oxide was also produced under the photochemical oxygen transfer conditions. The photochemical oxygen transfer not only hydroxylated aromatics, it also hydroxylated aliphatic compounds to carbinols and formed an epoxide from an olefin (see previous section). Thus, the photochemical transfer of oxygen from pyridine N-oxide to a substrate is a good model system for the microsomal hydroxylase system. The question which still remains is what is the nature of the active species of oxygen formed during irradiation of pyridine N-oxide. It is possible that the N-O bond breaks directly, forming atomic oxygen which reacts with the substrate; or that an intermediate oxaziridine species is formed which interacts with the substrate with subsequent oxidation of the substrate.

The question which I asked myself was whether the same intermediate, producing oxygen transfer, which is formed on irradiation of pyridine N-oxide, can be formed by the microsomal hydroxylase system. This active intermediate from the N-oxide could then be directed by the enzyme to the substrate (explaining the stereospecificity). The aromatic N-oxide (some unspecified N-oxide*) could be formed from the aromatic heterocyclic compound and oxygen. This possibility is explored in the second part of this thesis.

*The particular aromatic N-oxide studied was nicotinamide N-oxide. The reasons for choosing nicotinamide as the aromatic heterocyclic compound will be discussed in the second part of the thesis.

In the next short section, a few non-enzymatic, exploratory experiments are described.

Attempts to produce oxygen transfer from aromatic N-oxides by non-photochemical means.

1. Thermal

The aromatic N-oxide (pyridine N-oxide or nicotinamide N-oxide) was placed in a sealed tube with benzene or cyclohexene (10 mg N-oxide/ml solvent). The sealed tube was heated in a bomb at 350°C from 1 to 5 days. The resultant mixture was analyzed by glc for oxygenated solvent. There was no phenol (from benzene) nor cyclohexene oxide or cyclohexanone (from cyclohexene) formed in any of the tubes. Interestingly, when nicotinamide N-oxide was heated for 3 days, 3-cyanopyridine was formed. This product was identified by coinjection with authentic 3-cyanopyridine on a glc column and by comparison of the IR of the product with that of authentic 3-cyanopyridine.

2. Attempted oxygen transfer from aromatic N-oxides to anisole catalyzed by reduced metals

Ferris et al. (1968) found that tertiary amine oxides will rearrange in the presence of iron, Fe(II). This reaction is depicted below:

From the above results, the question arose whether reduced metals could catalyze deoxygenation (and thus oxygen transfer) of aromatic N-oxides. Since model hydroxylase systems employ reduced metals as catalysts to

activate molecular oxygen, the possibility that aromatic N-oxides could act as the oxygen source in these model systems was explored.

Several model hydroxylation systems were tried (see V. Ullrich and H. Staudinger, 1969, and ref. therein): ($\text{Fe}^{+2}/\text{EDTA}$; $\text{Fe}^{+2}/\text{EDTA}/\text{ascorbate}$; $\text{Sn}^{+2}/\text{HPO}_4^{-2}$.) The substrate used was anisole. A typical tube contained 5 micromoles reduced metal, 15 micromoles EDTA, 0.6 ml acetate buffer (0.1 M, pH 5.8), 10 μl anisole in 0.2 ml acetonitrile. The solutions were deoxygenated by bubbling helium through for an hour. Nicotinamide N-oxide or pyridine N-oxide were then introduced at various concentrations (5, 20, and 50 mM). The solution, after heating for an hour (temp. from 20 to 80°C were tried), was extracted with ether; the ether extracts were dried and washed; and the solution was analyzed by glc (3% OV-17 on Gas Chrom Q, Applied Sciences) for oxygenated anisole.

In all cases, under anaerobic conditions, whether an aromatic N-oxide was present or not, no hydroxylated product could be detected.

II-1 INTRODUCTION

Introduction

In this section I would like to first broadly sketch the characteristics of hydroxylase enzymes. I would then like to discuss microsomal hydroxylase systems, first placing these enzymes in the framework of future significant research and then discussing the mechanistic aspects of their action, thus introducing the specific problem of this thesis.

The central question of this thesis is: Can nicotinamide N-oxide act as a general oxygen donor in hydroxylase enzymes? Another way of asking the question would be whether nicotinamide N-oxide acts as a discrete intermediate in the incorporation of molecular oxygen into a substrate by a hydroxylase enzyme? Two major areas are incorporated in this question: 1) the biological role of aromatic N-oxides and 2) the mechanism of hydroxylase enzymes. The area concerning the role of aromatic N-oxides in biological systems has been recently reviewed by M. Bickel (1969). (This review also discusses aliphatic N-oxides.) My approach to the question concerning the relationship between N-oxides and hydroxylase systems is from the view of the mechanism of action of hydroxylases. The two hydroxylase systems looked at were the "microsomal hydroxylases" and microbial hydroxylases.

Hydroxylases

O. Hayaishi (1969) has written an excellent review concerning hydroxylase enzymes. He discusses the broad field of hydroxylases and gives many good leading references. I will only discuss a few aspects of hydroxylases, which are necessary for the main question.

In general, very few hydroxylases, have been purified and characterized. However, there seem to be several different mechanisms of introducing a hydroxyl group into substrate.

The hydroxylases systems which will be discussed in this thesis are the microsomal hydroxylases (squalene oxidase and laurate hydroxylase) and microbial hydroxylase (p-hydroxybenzoate hydroxylase). These hydroxylases are a subgroup of the class of ~~oxygenases~~ which are enzymes catalyzing the direct incorporation of molecular oxygen into a substrate. A reduced cofactor (NADPH) is required. One atom of the oxygen molecule is incorporated into the substrate and the other atom goes into water. The general requirements of the reaction are depicted below: S is the substrate, and E is the hydroxylase enzyme:

(It would be extremely interesting if this reaction were reversible to any measurable degree. There is no evidence reported for the reverse reaction.) These hydroxylations are "mixed function oxidases" according to the H. Mason (1957) definition or "external monooxygenases" according to the O. Hayaishi and M. Nozaki (1969) definition.

Microsomal Hydroxylation

The microsomal hydroxylase system (I call it a system since it contains an electron transport chain, see mechanism section) is contained in the microsomes which are vesicular sections of endoplasmic reticulum. These microsomes are formed spontaneously when the cells are disrupted by the process of homogenation. The microsomes

are isolated by differential centrifugation. It must be stressed that such an enzyme preparation is heterogeneous both enzymatically and physically. (There are "rough" microsomes containing ribosomes and "smooth" microsomes lacking ribosomes. The size and yield of microsomal particles is dependant on the method of homogenation.) It is not known whether there is a single, non-specific enzyme hydroxylating the various substrates or several more specific enzymes. It has proven difficult to characterize the enzyme system since it is integrally bound to the membrane.

The microsomal hydroxylase system is of paramount importance in metabolism of various substrates such as steroids and drugs. The role of these hydroxylases seems to be expanding continually. For example, R. Tenhunen, H. Marver, and R. Schmid (1969) have found that the microsomal hydroxylase system plays an important role in the breakdown of hemoglobin. In the section below, I would like to point up several major areas of research where these microsomal hydroxylase systems play an important role in the breakdown of hemoglobin. In the section below, I would like to point up several major areas of research where these microsomal hydroxylase systems may play an essential role. The understanding of these systems may lead to a new understanding of cellular control processes and possibly ability to modify these processes.

Significance Of Microsomal Hydroxylases

1. It is in the endoplasmic reticulum where substances from the external environment (drugs, food, toxic substances) many times first are acted upon. Most foreign substances entering the body are

"detoxified" in the endoplasmic reticulum by the hydroxylating enzymes. Hydroxylating renders the compounds soluble so that they can be excreted or further metabolized. The various aspects of drug oxidation by microsomes are discussed in "Microsomes and Drug Oxidation" (Gillette, 1969). It must be noted here that drugs induce the formation of endoplasmic reticulum with a concomitant increase in hydroxylase activity. This effect may be similar to induction of hepatic tyrosine- α -ketoglutarate transaminase by hydrocortisone (F. Kenney, D. Holten, W. Albritton, 1967). Steroids are implicated in the hydroxylase induction by S. Orrenius et al (1967). A definitive characterization of microsomal hydroxylase systems may provide a key to drug action and lead to modifying or specifying drug action by manipulating the hydroxylase enzymes or manipulating the structure of the drugs. These hydroxylase systems are of great importance today with the concern about the biological effects of pollution. (It is precisely the failure of microsomal hydroxylase to hydroxylate DDT that creates such problems with this insecticide.)

2. These hydroxylating enzymes metabolize the carcinogenic agents. Possibly, it is this hydroxylation reaction which may produce intermediates which cause the carcinogenic process to begin. It has been found that the carcinogenic process is an electrophilic type, which fits the hydroxylation process. (see sect. II). P. Grover and P. Sims (1968) and G. Gelboin (1969) found that carcinogenic aromatic hydrocarbons were chemically bound to DNA in the presence of microsomes.

Thus chemical carcinogenesis may be a direct result of the functioning of the microsomal hydroxylases. Specific chemical evidence for the mechanism of this action of the carcinogenic hydrocarbons with microsomes will be published shortly by E. Cavalieri (from this laboratory). I am indebted to E. Cavalieri for discussions on chemical carcinogenesis.

3. Since the microsomal hydroxylase system is so important in "cleaning up any garbage we might ingest," the functioning of these systems may play an important role in the aging process. One can imagine that if the functioning of these hydroxylating systems were impaired due to some external factors (such as the amount of various foreign materials ingested during a lifetime) or due to inherent changes within the cell (possible due to mistakes in DNA), the organism would not be able to cope with toxic substances and die. There is some evidence presented by W. Mainwaring (1969) suggesting that the endoplasmic reticulum is involved in the aging process in the mouse. He found that several proteins were absent in the endoplasmic reticulum of old mice relative to the proteins present in young mice.

Mechanism of Microsomal Hydroxylation

P. Strittmatter (1968), in his review on microsomal hydroxylations, provides a thorough discussion of the various components of the microsomal hydroxylase system. The scheme below provides the generally accepted mechanism for hydroxylation by microsomes:

The role of the pigment, cytochrome P-450, as the enzyme which interacts with molecular oxygen and substrate to produce the hydroxylated substrate has been definitively shown by T. Omura, et al. (1965). The cytochrome P-450 has been characterized by Y Ichikawa and T. Yamano (1970). The fact that the electron transport chain is essential to hydroxylase activity (as opposed to the direct reduction of oxygen by NADPH) was shown by J. Holtzman (1970). This general scheme, however, does not hold true in all cases of microsomal hydroxylations (Hayaishi, 1969) (Strittmatter, 1968). Speculation of how the cytochrome P₄₅₀ could activate the oxygen is discussed by H. Hill, A. Roder, and R. J. Williams (1970).

The overall reaction (oxidation of substrate by molecular oxygen) is a two electron oxidation. The iron of the cytochrome P-450 is only a one electron transfer center. H. Hill, et al (1970) suggests that a thiol, a ligand of the iron in the cytochrome, would provide the cytochrome, would provide the other electron in producing an O_2^{2-} as the hydroxylating species. R. J. Williams (1969) discusses the role of organic side chains in electron transfer centers. The one electron

transport properties of the iron in cytochrome P-450 may provide a key in understanding the carcinogenic properties of aromatic amines. (A carbon radical may be produced by the hydroxylase, E. Arrhenius, 1969).

The Role Of Nicotinamide In Oxygen Activation

One of the major questions which is still unanswered is: How is the oxygen "activated" by the enzyme in order to break the O-O double bond? From evidence presented in the section on model systems, some form of electrophilic oxygen is required (possibly OH^+). The following evidence suggests the possibility that nicotinamide N-oxide formed from nicotinamide may be involved in the formation of such an electrophilic oxygen in microsomal hydroxylations. The suggestion here is that molecular oxygen is activated by first producing nicotinamide N-oxide which in turn hydroxylates the substrate. The following five lines of evidence point to this possibility:

1. From the photochemical studies of aromatic N-oxides, it seems that there is an intermediate formed from the photochemical reaction which could produce an electrophilic oxygen species (OH^+). This same intermediate which should transfer oxygen in the photochemical system could be formed by the enzymatic system. The speculation of what this intermediate is has been discussed in the photochemical section. Particular note must be made that the photochemical hydroxylation of aromatic substrates produced the "NIH shift" to the same degree as microsomal hydroxylations. (D Jerina et al, 1970)
2. There is a direct transfer of oxygen from nicotinamide N-oxide to xanthine catalyzed by xanthine oxidase as shown on the following page.

(Murray, K. N. and Chaykin, S., 1966)

It must be noted that the mechanism of xanthine oxidase is not clearly understood. Generally xanthine oxidase acts as a dehydrogenase and does not fall in the category of a monooxygenase. Xanthine oxidase has been shown to also reduce a number of purine N-oxides, (G. Stohrer, 1968). Other N-oxides may also act as oxygen donors.

3. There is two to ten fold increase of the hydroxylase activity when nicotinamide is added to the liver homogenates. One example is a report by B. Brodie et al. (1955). Also see the results reported in the squalene oxidase section of this thesis. These increases in hydroxylase activity may not be general since some inhibition of hydroxylase activity was reported by J. Schenkman, J. Ball and R. Estabrook (1967). (However, these inhibitory results may be due to the particular conditions used).

4. A nitrogen atom in an aromatic ring such as pyridine can act as a site for catalytically transferring electrophilic species. A Fersht and W. Jencks (1969) reported evidence for an acetylpyridium ion intermediate in pyridine-catalyzed acyl transfer. The authors studied pyridine

catalyzed acylation of anisidine by acetic anhydride.

5. Along the same line of reasoning as in point 4, Albert Bobst (1968) found that in a cation radical of the pteridine series a substituent on N-5 had a very high group transfer potential. The substituent on N-5 would leave as a cation. Molecular orbital calculations were consistent with experimental group transfer potentials. An OH^+ is postulated as a possible group which is transferred. The structures of the pteridine is given below

Therefore, it seems a distinct possibility from the above evidence that nicotinamide N-oxide could possibly act as an oxygen transfer agent in microsomal hydroxylases. The following scheme presented in Fig. 1 is then proposed as an extension of the general scheme for microsomal hydroxylation proposed above. This scheme incorporates nicotinamide N-oxide as an intermediate formed in "activation" of molecular oxygen. Only cytochrome P450 is shown as being reduced by the NADPH. The intervening electron transport chain component is left out for simplicity.

If this proposed scheme is true, two major questions arise: 1) Is nicotinamide N-oxide formed enzymatically in the liver? and 2) Does nicotinamide N-oxide transfer its oxygen to the substrate? These two

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Figure 1. Proposed scheme for incorporation of nicotinamide N-oxide as an oxygen activating intermediate into microsomal hydroxylations. See text for details.

questions will be explored in subsequent sections of the thesis.

Once it is established that nicotinamide N-oxide is formed in the liver, the problem of choosing enzyme systems to test the oxygen transfer scheme arises.

Choosing The Enzyme System

Two enzymes were tried: The squalene oxidase and the free fatty acid hydroxylase. Microsomal hydroxylase systems are only very roughly characterized, and only recently have any of the components been solubilized. It seems that they are very tightly integrated with the membrane structure of the endoplasmic reticulum. Thus in both enzyme systems only the crudest enzyme preparations were attempted, in hope of gaining insight into the mechanism, with subsequent exploration into purification of the enzyme. Crude rat liver homogenates were used. The only purification which was done was by centrifugation which will be described in detail. Squalene oxidase was chosen since it closely paralleled the oxygen transfer reaction on the photochemical model system (epoxidation of a double bond). Also there was a very definite increase in oxidase activity with the addition of nicotinamide (see T. Chen and K. Block (1957) and results reported in squalene oxidase section). Laurate hydroxylase was chosen for two reasons: 1) A. Lu and M. Coon (1968) had succeeded in solubilizing some of the components of this hydroxylase system. They found that a low molecular weight, heat stable component was needed for hydroxylase activity. This small molecule could possibly be nicotinamide or some other aromatic heterocyclic compound. 2) Lauric acid had a marked effect on the reduction of nicotinamide N-oxide

(see Fig. 1 in the section under N-oxidase dealing with the reduction of nicotinamide N-oxide).

A third enzyme system was looked at since both of the above enzymes were membrane bound. This third enzyme studied was the p-hydroxybenzoate hydroxylase from psudomonas. This enzyme has been purified and characterized by K. Hosokawa and R. Stanier (1966). Thus this enzyme affords a soluble, pure enzyme for determining if possibly nicotinamide or its N-oxide had any effect on such a hydroxylase. The microbial hydroxylase enzymes differ from the microsomal hydroxylases as can be seen in the following brief summary of the mechanism of action.

The Microbial Hydroxylase: p-Hydroxybenzoate Hydroxylase

The microbial hydroxylase enzyme seems to be uncomplicated by electron transport components or membranes. The hydroxylation reaction of the benzoate requires NADPH, oxygen, and an FAD on the enzyme. The enzyme seems to be a single protein. The following scheme proposed by B. Hesp, M. Calvin, K. Hosokawa (1969) delineates the steps in hydroxylation.

This same scheme has been substantiated by S. Takemoro, et al, (1969). In this scheme too, the activation of molecular oxygen is not explained.

Nicotinamide is much more difficult to incorporate in any oxygen activation step since there does not exist any evidence in such microbial hydroxylation for nicotinamide participation. However, it was worth a try to see if nicotinamide had any effect on hydroxylase activity or if nicotinamide N-oxide would transfer oxygen to POB (p-hydroxybenzoate).

II-2 NICOTINAMIDE N-OXIDASE

Nicotinamide N-Oxidase

Introduction

From the discussion on the possible role of nicotinamide N-oxide in oxygen activation, it can be seen that there must exist a nicotinamide N-oxidase. Such an enzyme was found to exist in the rat liver and brain. The characteristics of this oxidase system were studied, using rat liver homogenates as the source of the enzyme. In the first section (A), the formation of nicotinamide N-oxide from nicotinamide will be described. In the second section (B), the reduction of nicotinamide N-oxide will be discussed.

A. Enzymatic Formation of Nicotinamide N-Oxide

Experimental

Materials

Nicotinamide-7-¹⁴C was purchased from New England Nuclear. The specific activity was 48 mC/mM. Nicotinamide, NADP, and glucose-6-phosphate (G-6-P) were purchased from Mann Biochemical. G-6-P dehydrogenase (1,000 IU/ml) was purchased from Cal Biochem. The rats used were Sprague-Dawley, male albino rats.

Enzyme preparation

Livers from at least two rats were homogenized and pooled. The 0.08 M phosphate buffer (pH 7.4) contained 1 mM Mg⁺⁺. The homogenates were then fractionated by centrifugation (see Figure 1 in the squalene oxidase section). The microsomal fraction, or P₁₀₀, is prepared by resuspending the pellet obtained from centrifugation of S₂₅ for 1 hr at 10⁵ g two times in the same volume of buffer and centrifuging at 10⁵ g again. The pellet is then suspended in buffer using a small

homogenator. Protein concentrations were determined in several experiments using the Biuret method. The S_{25} fraction contained approximately 60 mg protein/ml. The P_{100} fraction contained approximately 40 mg protein/ml. The S_{100} fraction contained approximately 15 mg protein/ml. These protein concentrations were not standardized from experiment to experiment, so that there is some variance in the N-oxidase activity. However, the results from a given pooled homogenate are self-consistent.

Incubations

A typical incubation contained: 2.0 ml liver homogenate; 0.4 ml NADPH generating system consisting of 0.6 ml of 15 mM NADP; 0.075 ml G-6-P; 0.015 ml G-6-P dehydrogenase; 0.2 ml nicotinamide ^{14}C solution (final conc. of 5 mM and 5 mC/mM). The tubes were incubated at 37°C with shaking. The enzymatic reaction was terminated by adding a 0.25 ml aliquot of the reaction mixture to 2 ml methanol. The precipitate was washed an additional three times with 2 ml methanol. The combined methanol washings were evaporated to dryness and 0.5 ml water was added (the flask was scratched with a spatula to suspend the small amount of precipitate). One-tenth ml of the water solution was spotted on Whatman No. 1 paper, and 100 μg nicotinamide N-oxide was added as carrier. The paper was developed in two dimensions. The first dimension was developed with 3% acetic acid in water saturated n-butanol. The second dimension was developed using n-butanol saturated with 3% ammonium hydroxide solution. The nicotinamide N-oxide was visualized by fluorescence under UV light. The radioactive spots

were visualized by exposure of the paper to film. The radioactive spots were counted using a hand geiger tube. The geiger tube was a flow type utilizing 99.05% helium and 0.95% isobutane. The efficiency for carbon-14 was approximately 15%. Most of the results are reported in counts per minute. Three thousand cpm is equivalent to approximately 1% conversion of nicotinamide to nicotinamide N-oxide. This figure was checked by eluting the radioactive spots with water and measuring the radioactivity using a liquid scintillation counter.

Results

The production of nicotinamide N-oxide from nicotinamide was verified by extracting the radioactive spot corresponding to nicotinamide N-oxide, adding carrier nicotinamide N-oxide, and measuring the specific activity. The nicotinamide N-oxide was then recrystallized three times from water, methanol, and water-ethanol. The specific activity for the three recrystallizations was 986 dpm/gm, 998 dpm/gm, 996 dpm/gm.

The quantitative determination of nicotinamide N-oxide is complicated by the fact that during the procedure for extracting, separating, and purifying nicotinamide N-oxide, the N-oxide reverts to some degree to nicotinamide. See Section B on the reduction of nicotinamide N-oxide.

Effect of Cofactors on the Formation of Nicotinamide N-Oxide

Figure 1 shows the effect of NADPH (as a NADPH generating system) on the formation of nicotinamide N-oxide. Twenty-five hundredths ml

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Figure 1.

aliquots were removed at various times during the course of the incubation and assayed for the nicotinamide N-oxide content.

The formation of nicotinamide N-oxide was examined under aerobic and anaerobic conditions. The results are reported in Figures 2 and 3. In Figure 2 the formation of nicotinamide N-oxide by the S_1 fraction of liver homogenates is shown as a function of time. The incubation mixture is the one described under incubations in the experimental section.

Oxygen or helium respectively were flushed over the top of the reaction vessel for several minutes and bubbled through the homogenate. A small positive pressure of the gas was maintained over the top of the solution. Twenty-five hundredths ml aliquots were removed at the specified times and assayed for nicotinamide N-oxide.

In another experiment with the S_1 fraction, using Thunberg tubes and incubation times of 2 hr, the cpm in nicotinamide N-oxide from tubes incubated under helium, air, and oxygen was found to be 5,500, 8,000, and 9,000.

In Figure 3, the formation of nicotinamide N-oxide by the P_{100} fraction (twice washed) was examined under various atmospheres. In this case, each point on the graph represents a separate reaction vessel which was terminated at the specified time. The reaction vessels were Thunberg tubes which were evacuated and flushed with the specified gas. The homogenate was placed in the top compartment and the nicotinamide with the NADPH generating system was placed in the bottom compartment. The tubes were tipped and incubated at 37°C.

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Figure 2. The formation of nicotine N-oxide by liver homogenates (S₁ fraction). Three tubes (described in text) were incubated with C¹⁴ nicotine during the course of incubation and assayed for nicotine N-oxide. The ordinate axis is the cpm in the nicotine N-oxide.

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Figure 3. The formation of nicotinamide N-oxide by liver homogenates (P₁₀₀ fraction). Each incubation tube (described in text) was terminated and assayed for nicotinamide N-oxide at the time indicated. The ordinate axis refers to the cpm in the nicotinamide N-oxide. The thunberg tubes were flushed with the gasses indicated.

Purification of the Nicotinamide N-Oxidase

The first purification step was fractionation of the liver homogenate. Table 1 shows the activity of the nicotinamide N-oxidase in various fractions of the homogenate. It is evident from this data that there is a soluble component of the N-oxidase which is a protein. Note that the potentiating effect on N-oxidase activity in P_{100} by the addition of S_{100} is destroyed by boiling the S_{100} .

There were a number of attempts to solubilize the N-oxidase from the microsomal fraction. All attempts failed. Freeze-thaw, sonication, and digitonin simply did not solubilize any of the N-oxidase. Deoxycholate (0.5%) or Triton X (1%) treatment destroyed enzymatic activity. Addition of dithiothreitol (0.1 M) also destroyed the enzymatic activity.

The procedure in attempting to solubilize the N-oxidase is described below. Two methods were tried: 1) adding the detergent or applying the manipulation to the S_{25} fraction, centrifugation at 10^6 g and assaying the S_{100} for an increase in activity; and 2) resuspending the pellet (P_{100}) in the detergent solution, centrifugation at 10^5 g and assaying the S_{100} and the P_{100} for activity.

Formation of nicotinamide N-oxide by rat brain

Four rat brains (total weight 9 g) were excised from decapitated rats and homogenated with 22 ml buffer. The fractionation and incubation procedures were the same as those used with rat liver. The incubation time was 2 hr.

Table 1

NICOTINAMIDE-N-OXIDE FORMATION BY DIFFERENT FRACTIONS OF
RAT-LIVER HOMOGENATES

Liver Homogenate fraction	cpm in Nicotinamide-N-Oxide		
	exp. (i)	exp. (ii)	exp. (iii)
Boiled S ₂₅	700		
S ₁	7,800		
S ₂₅	8,000		3,600
S ₁₀₀	700	1,500	950
P ₁₀₀	3,600	7,000	2,000
P ₁₀₀ + S ₁₀₀	6,000	16,500	4,000
P ₁₀₀ + Boiled S ₁₀₀		5,000	2,100

NOTE: The cpm reported is an average of duplicate runs on the same homogenate. In exp. iii, 5 volumes/gram liver were used in the homogenation instead of the usual 2.5 volumes buffer/gram liver.

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<u>Fraction</u>	<u>cpm in nicotinamide N-oxide</u>
boiled S ₁	580
S ₁	950
S ₁₀₀	790
P ₁₀₀	1470

Formation of nicotinamide N-oxide by acetone powder

Acetone powder of rat liver and rat brain were obtained from Mann Biochemical. 0.4 g were suspended in 10 ml buffer. The incubation procedure was the same as on fresh homogenates. The incubation was terminated at the end of 2 hr. The following are the results:

<u>Fraction</u>	<u>cpm in nicotinamide N-oxide</u>
brain	4,300
boiled brain	1,000
liver	4,400
boiled liver	1,000

Check for nicotinamide N-oxidase in other organisms

E. coli, yeast, and spinach leaves were assayed for nicotinamide N-oxidase activity. There was no N-oxidase activity in any of these organisms.

Procedure:

E. coli: 100 ml of E. coli (20 mg protein/ml) was spun down at 3,000 g. The bugs were resuspended in 10 ml buffer medium and put through the french press two times. The incubation procedure was the same as for liver homogenates, except that the temperature was 30°C.

Yeast: Two grams frozen yeast was suspended in 10 ml buffer and put through the french press. The assay for N-oxidase was the same as for E. coli.

Spinach leaves: Fresh leaves were picked off a plant, washed, cooled, ribbed. 15 grams were homogenized with 30 ml buffer. The homogenate was fractionated the same as the liver homogenate, and the incubations were the same. S_0 , S_1 , S_{25} , S_{100} , and P_{100} were tried.

Discussion

The important result which is essential to the main question concerning the role of nicotinamide N-oxide in hydroxylase systems is that there is an enzyme in the microsomes which converts nicotinamide to its N-oxide.

There are a number of interesting questions which arise from the data obtained. However, it must be noted that the enzyme system is very crude and any definitive answers must wait until the system is solubilized and purified.

There are several factors which implicate the microsomal hydroxylase system in the oxidation of nicotinamide. (A most important fact concerning the source of the source of the oxygen will have to wait for isotopic labelling studies.) The N-oxidase activity is destroyed by detergents. Cytochrome P_{450} is converted to inactive cytochrome P_{420} by the addition of detergents (T. Omura and R. Sato, 1964). J. Schenkman et al. (1967) found that addition of nicotinamide to microsomes in the presence of hydroxylase substrates produced absorption spectra changes. This indicates that the nicotinamide

binds to the microsomes. In this same paper the authors report that nicotinamide is a competitive inhibitor of aminopyrine demethylase. The significance of this, particularly in light of reported increases in hydroxylase activity with the addition of nicotinamide, is puzzling.

The formation of nicotinamide N-oxide under anaerobic conditions is puzzling. From Figure 3, where each point is a separate tube, no N-oxide is produced for the first hour. It is possible that there was incomplete deoxygenation, due to the procedure used. Barrie Hesp (from this lab), in his experiments with deoxygenating enzyme solutions, found that only after extensive bubbling of the solution with helium was the oxygen removed. (Evacuation and flushing with He was not sufficient.) Another possible explanation for the results is that there is an activation of another hydroxylase (possibly dehydrogenase) system under anaerobic conditions. It must be noted that from the experiments of J. Schenkman et al. (1967), it was found that NADPH dependent hydroxylases lost their activity very rapidly with time. (Note that these were aerobic incubations.) Differential inactivation or activation of various enzyme systems or components of these enzyme systems, could possibly explain results as obtained in Figure 2. The conclusion of this little exercise is that it is highly speculative to conclude anything about fine mechanistic details from a crude system of unknown composition and structure.

Conclusion

Oxidation of a nitrogen atom to make N-oxides is a general biochemical reaction (M. Bickel, 1969). J. Kirchner and S. Chaykin (1963) reported that nicotinamide N-oxide was formed from nicotinamide in the liver. We have confirmed this finding, extending the scope of the enzyme system.

B. Reduction of Nicotinamide N-Oxide

There has been much confusion in the literature whether the reduction of N-oxides are enzymatic, non-enzymatic, or require co-factors (M. Bickel, 1969, p. 341). This same anomalous behavior has been observed in the case of nicotinamide N-oxide, with the microsomal fraction of the liver homogenates. At first the reduction of nicotinamide N-oxide was observed to be affected by various substrates. Then it was discovered that nicotinamide N-oxide was reduced even in the absence of any enzyme. An investigation was undertaken as to the source of this reduction. It was established that reduction occurred during the work-up of the reaction. The particular experiments and the results will be discussed below.

Procedure

The same incubation procedure was used as with the formation of the nicotinamide N-O. Also the same extraction procedure was used. However, only one-dimensional paper chromatography was employed using the basic solvent to develop the paper. In all the following experiments where P_{100} is noted, a twice washed microsomal fraction of rat liver homogenate was used. (A washing of the microsomal fraction consists of resuspending the pellet (P_{100}), in buffer, and

centrifuging again to make washed P_{100} .) In all the incubations ^{14}C nicotinamide N-oxide ($4 \mu\text{C}/\mu\text{M}$) was used in a final concentration of 0.5 mM. The results are reported in cpm, since all sample sizes and activities were the same. In the radioautography 3,000 cpm is equivalent to approximately 1% conversion of nicotinamide N-oxide to nicotinamide.

Results

In Table 1 the effect of various substrates of the microsomal hydroxylase system on the reduction of nicotinamide N-oxide are shown. These substrates were present in a concentration of 5 mM. In comparing the effect of these various compounds, it seems that lauric acid has the greatest effect. However, it can be noted that the blanks contained a considerable amount of nicotinamide. In Table 2 the effect of lauric acid and cofactors was explored. Here it can be seen that the blanks contained very little nicotinamide. The presence of NADPH seems to increase the amount of nicotinamide formed. In Table 3 further controls were done. It can be seen that the presence of NADPH again increases the amount of nicotinamide formed. The surprising result was that nicotinamide N-oxide alone with buffer was able to be reduced to a substantial degree (approx. 7%). The degree of reduction seems to be greater than any previous reduction in the presence of liver homogenate.

The control experiments presented in Table 3 indicate that the isolation and separation procedure may yield some reduction of the N-oxide. The chromatographic procedure itself was easily checked by simply spotting a sample of nicotinamide N-oxide directly from

Table 1

Incubation of C¹⁴ Nicotinamide N-Oxide with Liver Microsomes
Effect of Drugs

<u>Drug (5 mM)</u>	<u>cpm in nicotinamide</u>
aminopyrine	2,600
aniline	2,100
caffeine	2,900
codeine	2,500
squalene	3,400
xanthine	6,900
lauric acid	11,600
blank	8,400

Table 2

Incubation of C¹⁴ Nicotinamide N-Oxide with Liver Microsomes

<u>NADPH*</u>	<u>Laurate (10 mM)</u>	<u>He/air</u>	<u>cpm in nicotinamide</u>
+	+	He	7,400
+	-	He	9,700
-	+	He	2,400
-	-	He	1,200
+	+	Air	12,600
+	-	Air	5,000
-	+	Air	1,500
-	-	Air	1,600

Table 3

Reduction of Nicotinamide N-Oxide

<u>Cofactor</u>	<u>Liver homogenate</u>	<u>cpm in nicotinamide</u>
NADPH (10 mM)	P ₁₀₀	10,500
NADP (10 mM)	P ₁₀₀	4,200
---	P ₁₀₀	6,500
NADPH (10 mM)	---	26,000
---	---	20,000

the stock solution, developing the paper, and radioautography.

There was less than 0.5% nicotinamide formed.

An attempt was made to determine the amount of nicotinamide formed without separating the isolating the products. The first experiment, the reduction of nicotinamide N-oxide by NADPH, was followed using the UV absorption band at 340 to monitor the amount of NADPH. There was no change in the O.D. at 340, indicating the NADPH did not reduce the nicotinamide N-oxide. Another method of determining the conversion of nicotinamide N-oxide to nicotinamide in solution was to use a colimetric determination of nicotinamide developed by Mueller and Fox (1951). On incubation of a solution of nicotinamide N-oxide in buffer, with or without added NADPH, NADP, NAD, NADH, no nicotinamide was produced. However, if the extraction procedure was used (i.e., combining methanol fractions and evaporating under reduced vacuum to dryness), nicotinamide was produced in approximately 3% of the N-oxide added.

Conclusion

The experimental evidence presented here indicates very strongly that there is some reduction of nicotinamide N-oxide by a non-enzymatic process. This non-enzymatic reduction may be caused by NADPH. However, until a procedure is developed which eliminates any reduction during the work-up and separation of nicotinamide N-oxide from nicotinamide, it will be impossible to determine the extent of enzymatic reduction of nicotinamide N-oxide.

Two trends emerge which would need clear definition: 1) The presence of NADPH increases the amount of nicotinamide formed

(Figs. 2 and 3; and 2) the presence of microsomal fraction, irrespective of added cofactors, seems to reduce substantially the amount of nicotinamide formed. (Compare especially the 1500-6500 cpm in nicotinamide when P_{100} is incubated with nicotinamide N-oxide with the 20,000 cpm in nicotinamide when only nicotinamide N-oxide is incubated in buffer.)

The possibility remains that the reported enzymatic reduction of aromatic N-oxides may be completely due to xanthine oxidase. K. Murray and S. Chaykin (1966) reported that xanthine oxidase reduces nicotinamide N-oxide. Recently G. Stohrer and G. Brown (1969) found that xanthine oxidase reduces a series of purine N-oxides.

II-3 SQUALENE OXIDASE

Squalene Oxidase

Introduction

In the last section it was shown that nicotinamide N-oxide is indeed a metabolite of nicotinamide. The question that is now posed is whether there is a physiological function for nicotinamide N-oxide, particularly in relation to oxygen activation in hydroxylase systems. One possible role for nicotinamide N-oxide has been outlined in the introduction (II-1). Whether nicotinamide N-oxide can act as a substitute for molecular oxygen is tested as described in the following sections. The three enzyme systems used are: 1) squalene oxidase, 2) laurate hydroxylase, and 3) p-hydroxybenzoate hydroxylase. The approach is to see if any oxygenated substrates are formed by these enzymes under anaerobic conditions in the presence of nicotinamide N-oxide.

In this section (II-3), squalene oxidase is examined. Squalene acts as the open chain precursor to the sterol structure. The biosynthesis of squalene and its subsequent cyclization to the sterol structure is reviewed by R. Clayton (1965). There was very much interest in the oxidation and cyclization of the squalene molecule, both from a chemical and biochemical viewpoint. In 1966, E. Corey et al. (1966) and E. Van Tamelen et al. (1966) independently showed that squalene 2,3-epoxide was the intermediate formed from squalene before cyclization took place. The cofactor requirements for the conversion of squalene to sterols were determined by T. Tchen and Bloch (1957) to be the same as those for other "mixed function" oxidases (O_2 , NADPH).

It was subsequently shown on a crude enzyme preparation (liver homogenate) by J. D. Willett et al. (1967) and on the purified "oxidosqualene sterol cyclase" (P. Dean et al., 1967), that the cyclization reaction did not require oxygen or NADPH as cofactors. Thus, the conversion of squalene to the epoxide is a mixed function oxidase. T. Tchen and K. Bloch (1957) had also shown that nicotinamide increased the production of sterols from squalene fourfold over the levels of sterols produced without nicotinamide.

Due to the interest and significance of squalene cyclization, due to the fact that squalene "epoxidase" is a mixed function oxidase, and due to the fact that nicotinamide is implicated in this reaction, squalene oxidase was chosen as one of the enzyme systems to study. Since it was shown that the cyclization of squalene epoxide does not depend on any cofactors and since squalene oxidase is in the microsomes, the squalene oxidase system can be studied by looking at the conversion of squalene to sterols by liver homogenates.

Experimental

A crude enzyme preparation was used consisting of fractions of rat liver homogenate. The enzyme was assayed by following the incorporation of radioactive labeled squalene into sterols. The compounds were separated by thin-layer chromatography (TLC) and gas-liquid chromatography (GLC).

Materials

DL Mevalonic acid-5-³H (50 mC/mM) as DBED salt was obtained from New England Nuclear. ATP, NADP, G-6-P, and G-6-P dehydrogenase were obtained from Cal Biochem. Squalene (Eastman Kodak) was purified by

gas-liquid chromatography (see assay procedures for conditions).

Lanosterol, cholesterol, and dihydrolanosterol were purified according to the procedures employed by Willet, et al. (1967). In the preparation of squalene oxide the squalene oxide prepared from the bromohydrin was further purified by column chromatography on silicic acid. The squalene oxide gave the same NMR, elemental analysis, IR, and chromatographic behavior as reported by Willet (1967).

Preparation of Liver Homogenates

The method of homogenation was the same as reported by Bucher and McGarrah (1956). All work was done at ice temperatures. Male albino rats were decapitated and their livers excised (liver weights averaged 15 g). Each liver was chopped up with a razor blade and homogenized (2.5 ml pH 7.4 phosphate buffer per gram liver) for 30 sec in a Potter-Elvehjem homogenizer equipped with a loose fitting teflon pestle (about 5 mm space between pestle and wall). The crude homogenate was immediately filtered through surgical gauze to remove large particles and fractionated by centrifugation. The fractionation is described in Figure 1.

Incubation of Homogenates

Reaction vessel: A round-bottom flat (15 ml volume) with a Thunberg joint was used. It was found that the shape of the reaction vessel was an essential factor in the incorporation of added squalene into sterols. A round-bottom flask with vigorous shaking gave the best incorporation compared to essentially no incorporation if a test tube type reaction vessel was used. This is probably due to the

FRACTIONATION OF LIVER HOMOGENATES

rat livers ground in Potter-Elvehjem homogenizer
(2.5 ml pH 7.4 phosphate buffer /g liver).

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Figure 1.

fact that squalene is essentially insoluble and therefore its availability to the enzyme system is due to dispersion of squalene and lipid droplets throughout the homogenate. It was noted that the crude homogenates contained discrete lipid droplets dispersed through the homogenate (possibly the squalene is dissolved in these lipid droplets). If future assays of this type are to be undertaken, two possible avenues of overcoming the solubility problem suggest themselves: 1) Add some detergent such as sodium dodecylsulfate (this method would fail if the oxidase is integrally dependent on its structural position in the membranes as many mixed function oxidase systems are); and 2) sonicate the entire incubation mix prior to warming, thus possibly dispersing the squalene in small enough particles so that they will remain suspended.

The Incubation

The incubation is carried out at 37° with shaking. Squalene is first added in a benzene solution on top of 10 μ propylene glycol. After the benzene is evaporated the squalene is in a film on the propylene glycol. The liver homogenate fraction is then added. The water soluble components are added in small aliquots. If anaerobic conditions are needed, the NADPH (or NADPH generating system) was placed in the top compartment of the Thunberg tube. The tube is evacuated to 0.4 mm Hg, then flushed with helium (this procedure is repeated three times). After anaerobic conditions are established, the Thunberg tube is tipped and the reaction initiated. A typical incubation mix consists of first adding 165 μ g squalene (5×10^5 dpm) and

20 λ propylene glycol. Three ml S_1 fraction (combined homogenates of at least two rats) was added, then 0.7 ml of a NADPH generating system (0.66 ml 1.5×10^{-2} M NADP, 0.1 ml 0.2 M G-6-P, 10 λ G-6-P dehydrogenase, 2.6 ml buffer). The reaction was terminated by addition of 3 volumes of a 2:1 methanol: 15% aqueous KOH solution. Carrier compounds were added in small volumes of acetone. The mixture was sonicated for 1 min and allowed to stand for 15 hr. After the completion of this saponification, the mixture was exhaustively extracted with hexane. Greater than 95% recovery of added radioactivity was achieved (less than 1% of counts remained in the aqueous layer). It was found that the best extraction procedure was to vigorously stir the saponification mix with an equal volume of hexane in a large centrifuge tube with a magnetic stirrer for 12 hr. After centrifugation, the hexane and aqueous layers were well separated and the hexane layer was drawn off with a pipette. It was found that simple shaking in a separatory flask was unsatisfactory, since after shaking three times with equal volumes of hexane only 60% of the activity was recovered. The hexane extracts were washed twice with water, dried with sodium sulfate, and the hexane evaporated. A small quantity of benzene was added and the solution was spotted on a TLC sheet or injected into GC.

TLC

ChromAR 500 (Mallinckrodt Chemical Co.) prepared TLC sheets were used. The sheets were developed with 2% ethyl acetate in n-hexane. A spot containing a mixture of pure compounds (squalene, squalene oxide, cholesterol and lanosterol) were placed on the edges of the sheet to

determine the positions. The sheet was cut vertically into strips and the edges visualized with 10% sulfuric acid. The middle strip (rf: squalene, 0.8; squalene oxide, 0.55; lanosterol, 0.2; cholesterol, 0.1) containing the sample spot was cut horizontally, corresponding to the various fractions and shredded directly into scintillation fluid and counted.

GLC

A glass column was used with on-column injection. The packing was 3% OV17 on GasChrom Q (Applied Science Lab). A sample of the chromatograph is shown in Figure 2. The fractions corresponding to the various peaks were collected in capillary tubes and washed with benzene into scintillation tubes and counted. Approximately 70% of the radioactivity injected in the GC was recovered. The collection efficiency was approximately 60 to 80% as measured by weighing collected fractions of injections of known quantity. The squalene oxide injected was quantitatively converted to two products. This thermal rearrangement will be described below.

Comparison between Results on TLC and GC

In both cases, the sample containing the radioactive squalene and its products was separated and the individual components counted. The data are presented in relative percent that each fraction had with respect to the total dpm collected. This method makes the assumption that the collection efficiencies are equal. When a comparison is made by splitting a particular sample in two parts and using the two chromatographic techniques on each part, the GC results are lower in the relative amount of sterols present. This may be due to some decomposition

XBL 694-4193

Figure 2. Separation of squalene, squalene oxide and sterols by gas liquid chromatography. Conditions: 6' x 1/4" glass column with on-column injection packed with 3% OV 17 on gaschrom. Q (Applied Science Lab); He gas flow 50 ml/min; inj. temp., 280°C; detector temp., 280°C. Peak identification: 1, squalene; 2 and 3, squalene oxide; 4, cholesterol; 5, dihydrolanosterol; 6, lanosterol.

of the sterols on the column, since the collection efficiencies are approximately equal. However, the comparison within one chromatographic procedure leads to the same conclusion for the experiments. Note also that the conversions of squalene to sterols varies widely; however, the results within a set of experiments give the same conclusion.

Thermal Rearrangement of Squalene Oxide

As can be seen in Figure 2, there are two peaks for squalene oxide (peaks numbered 2 and 3). These two peaks were each collected and purified by reinjection on the same column. The fact that squalene oxide quantitatively rearranged to these two products was shown in the following manner. A known quantity of squalene oxide was injected, and the areas under the product peaks were measured. These areas were compared to standard mass-area measurements made by injecting known quantities of each of the two purified products. The quantity of squalene oxide calculated from the comparison of areas corresponded closely (within 5%) to the amount actually injected. The results of the rearrangement are given in Figure 3. Compound A of Figure 3 corresponds to peak number 2 of Figure 2; and compound B corresponds to peak 3. The two products are identified by their NMR and IR. Both NMR and IR were taken in CCl_4 . The NMR data and IR data is tabulated in Tables 1 and 2. The assignments refer to the reaction as shown in Figure 3. In the IR spectra, the absorption maxima reported are those of new bands which are not present in the squalene epoxide IR.

THERMAL REARRANGEMENT OF SQUALENE-2,3-EPOXIDE

XBL 705-5196

Figure 3. The thermal rearrangement takes place on a GLC column as described in Figure 2. Peaks 2 and 3 of Figure 2 correspond to compounds A and B.

Table 1220 MHz NMR of Products of Squalene Epoxide RearrangementSqualene-2,3-epoxide

<u>Spectrum (δ)</u>	<u>Integration</u>	<u>Assignment</u>
1.18, 1.20	6 H	methyl H(a)
1.57, 1.59, 1.64	20 H	vinyl methyl + 2 H(b)
1.95	18 H	allylic H
2.48 (t, j=6)	1 H	H(c)
5.05	5 H	vinyl H

j = coupling constant

<u>A</u>			<u>B</u>		
<u>Spectrum (δ)</u>	<u>Integration</u>	<u>Assignment</u>	<u>Spectrum (δ)</u>	<u>Integration</u>	<u>Assignment</u>
1.02, 1.07	6 H	methyl H(a)	1.16, 1.25	2 H	methylene H(b)
1.57, 1.64	20 H	vinyl methyl + 2 H(b)	1.57, 1.64, 1.68	21 H	vinyl methyl H(a)
1.95	19 H	allylic H + 1 H(c)	1.98	18 H	allylic H
5.05	5 H	vinyl H	3.91 (t, j=6)	1 H	H(c)
			4.71	1 H	{ terminal vinyl H(d)
			4.84	1 H	
			5.05	5 H	vinyl H

Table 2IR of Products of Squalene Epoxide RearrangementSqualene-2,3-epoxide

<u>IR max</u>	<u>Assignment</u>
2960	ν_{as} CH ₃
2920	ν_{as} CH ₂
2845	ν_s CH ₂
1450	δ_{as} CH ₃
1380	δ_s CH ₃
1245	epoxide

A
(new bands appearing)

<u>IR max</u>	<u>Assignment</u>
1725	ν C=O
1715	
1375	
1380	C $\begin{matrix} \text{CH}_3 \\ \text{CH}_3 \end{matrix}$
doublet	
(no 1245)	

B
(new bands appearing)

<u>IR max</u>	<u>Assignment</u>
3620	O-H
1655	($\nu_{C=C}$)
890	(δ_{CH})
(no 1245)	

Preparation of Squalene ^3H

Three rats were sacrificed, and the livers were homogenated in 0.08 M phosphate buffer (pH 7.4) containing 4 mM MgCl_2 and 30 mM nicotinamide. A solution was made up containing 40 ml S_{25} fraction, 70 mg mevalonic acid (DBED salt), 0.25 mC 5- ^3H mevalonic acid (50 mc/mM), 10 mg ATP, and 7 ml NADPH generating system (1.33 ml 1.5×10^{-2} M NADP, 0.2 ml 0.25 M G-6-P, 0.01 ml G-6-P dehydrogenase, and 5.5 ml buffer).

Ten Thunberg tubes, each containing 4.8 ml of the stock solution were flushed with He by evacuation four times, and sealed under helium, and incubated at 37°C for 2 hr. The tubes were combined and the reaction was terminated by the addition of 3 volumes of a 2 to 1 methanol 15% KOH solution. The mixture was allowed to stand approx. 15 hr, at which time it was extracted three times with its own volume of hexane. The hexane fraction was washed two times with water and dried over sodium sulfate. The solution was evaporated and placed on an alumina column. The squalene was eluted with hexane. The activity of the eluted fraction containing squalene was 1.1×10^7 dpm. The hexane was evaporated and the squalene was made up to 1 ml with benzene and stored in the freezer.

Results

The following tables give the experimental results. Much work is not reported here since the experimental procedures were not yet standardized to give good yields of conversion of squalene to sterols. Even when the experimental procedures (note discussion on configuration of reaction vessel) were standardized, the conversion of squalene to

<u>SQ-K.</u>			
<u>Tube</u>	<u>Atmosphere</u>	<u>Propylene glycol</u>	<u>% Sterols (TLC)</u>
I	O ₂	10 λ	17
II	O ₂	100 λ	22
III	O ₂	300 λ	12

Each tube contained: 165 μ g squalene (10^6 dpm) and 3.7 ml of solution containing 15 ml S₁ liver homogenate (buffer contained 4 mM Mg⁺⁺ and 30 mM nicotinamide); 0.66 ml 1.5×10^{-2} M NADP; 0.1 ml 0.25 M G-6-P; 0.01 ml G-6-P dehydrogenase; and 2.6 ml buffer.

SQ-L

<u>Tube</u>	<u>Atmosphere</u>	<u>Nic N-O</u>	<u>Nic</u>	<u>% Sterols (TLC)</u>	<u>% Sterols (GC)</u>
I	O ₂	--	--	4	1.9
II	O ₂	--	40 mM	9.7	3.1
III	O ₂	40 mM	--	5.7	2.2
IV	He	--	--	3.4	1.3
VI	He	40 mM	--	3.4	1.2

Each tube contained: 3 ml S₁ liver homogenate (buffer contained 4 mM Mg⁺⁺); 0.08 ml propylene glycol; 165 µg squalene (10⁶ dpm); 0.13 ml 1.5 x 10⁻² M NADP; 0.02 ml 0.25 M G-6-P; 0.002 ml G-6-P dehydrogenase; 0.5 ml buffer.

<u>SQ-M</u>						
<u>Tube</u>	<u>Atmosphere</u>	<u>Nic W-0</u>	<u>% Squalene epoxide</u>		<u>% Sterols</u>	
			<u>TLC</u>	<u>(GC)</u>	<u>TLC</u>	<u>(GC)</u>
I	O ₂	--	2.4	(2.8)	7.3	(4.1)
II	O ₂	32 mM	1.5	(2.7)	6.3	(3.4)
III	He	--	1.2	(3.3)	4.6	(2.4)
IV	He	32 mM	0.8	(2.1)	2.3	(2.8)

Each tube contained: 4 ml S₁ liver homogenate (buffer contained 4 mM Mg⁺⁺);
 0.05 ml propylene glycol; 1.65 µg squalene (2 x 10⁵ dpm);
 1 mM NADPH; 0.5 mM NAD.

SQ-N

<u>Tube</u>	<u>Atmosphere</u>	<u>% Sterols (TLC)</u>
I	O ₂	7
II	He	2
III	O ₂	15
IV	He	1

Each tube contained: 4 ml S₁ liver homogenate (buffer contained 4 mM Mg⁺⁺ and 40 mM nicotinamide N-oxide); 0.05 ml propylene glycol; 165 µg squalene (see Note) (10⁶ dpm); 1 mM NADPH; and 0.5 mM NAD.

Note: Tubes III and IV had squalene ³H from a different batch than used in SQ-K, L, M and N (I and II).

<u>SQ-0</u>			
<u>Tube</u>	<u>Nicotinamide (mM)</u>	<u>Squalene (mM)</u>	<u>% Nicotiramide N-oxide</u>
I	5	0.0	0.73
II	5	0.1	0.81
III	5	0.5	0.82
IV	5	2.0	0.85
V	40	0.0	0.40
VI	40	0.1	0.44
VII	40	0.5	0.48
VIII	40	2.0	0.48

Each tube contained. (final volume 0.3 ml): 0.220 ml S₁ liver homogenate (buffer contained 4 mM Mg⁺⁺); 0.002 ml propylene glycol; 10 mM NADPH; 5 x 10⁶ dpm C¹⁴ in the nicotinamide.

sterols varied considerably. This may be due to changes in activity of the enzyme systems or to some uncontrollable variable such as dispersion of squalene in the homogenate. Note that in SQ-N a change in the batch of squalene ^3H made a considerable difference in the sterols produced under O_2 . Note that in SQ-K and L an NADPH generating system was used, whereas in SQ-M and N, NADPH was used. The concentration of NADPH was the same as Scallen et al. (1968).

Note that in SQ-N, the livers were homogenated in buffer containing nicotinamide N-oxide. This experiment was tried since there was a possibility that the site of action of the oxidase was on the inside of the microsomal vesicles and the N-oxide could not pass through the membrane.

In SQ-O, the relationship between the amount of squalene present and the amount of nicotinamide N-oxide formed was studied. This experiment was simply an exploratory one to see if there were any large changes indicating a strong coupling between squalene oxidation and nicotinamide N-oxide formation. Even though there seems to be some slight trend of increasing N-oxide formation with increasing amounts of squalene added, this does not seem to be significant in the light of the errors inherent in the procedure (as discussed in the section on nicotinamide N-oxidase). In this experiment the procedures used to determine the amount of nicotinamide N-oxide were similar to those described in Section II-2.

Conclusion

The major conclusion that can be drawn from these data is that nicotinamide N-oxide does not transfer its oxygen to squalene under anaerobic conditions. Thus, nicotinamide N-oxide cannot be a discrete intermediate in activation of oxygen by squalene oxidase.

II-4 LAURATE HYDROXYLASE

Free Fatty Acid Hydroxylase

Introduction

After the study on squalene oxidase presented in the previous section, a second microsomal mixed function oxidase system was studied. This second system is the hydroxylation of lauric acid. (Peterson et al., 1969, studied the stoichiometry.) This particular enzyme (or actually substrate for the microsomal enzyme system which may be a single, non-specific hydroxylase) was chosen for several reasons. Lauric acid showed an effect on the reduction of nicotinamide N-oxide in the presence of microsomes (see Table 1, section II-2). Hydroxylation in the ω and $\omega-1$ positions of long chain fatty acids had been demonstrated by B. Preiss and K. Bloch (1964). M. Kusunose et al. (1964) demonstrated ω -hydroxylation of lauric acid. A. Lu and M. Coon (1968) succeeded in solubilizing the enzyme system catalyzing ω -hydroxylation of lauric acid in rabbit liver microsomes. Three components of the enzyme system were required for activity: cytochrome P-450, TPNH-cytochrome c reductase, and a heat stable factor which was extractable into ether. The possibility that this heat stable factor could be nicotinamide or some heterocyclic aromatic compound supported the investigation of this enzyme system. The same general experimental approach was used as with the squalene oxidase: nicotinamide N-oxide was incubated with liver homogenate, NADPH, and laurate under aerobic and anaerobic conditions and the hydroxylated products were compared.

Experimental

A stock solution of C^{14} sodium laurate (purchased from New England Nuclear) was made up so that 20 ml contained 10^8 dpm and the concentration was 50 mM. 0.2 ml of this stock solution was used per 2 ml of the liver homogenate to make a final concentration in the incubation mixture of 0.5 mM of lauric acid. The NADPH generating system was the same one that was used in the squalene oxidase experiments. The homogenations were done as described in the squalene oxidase section.

A typical incubation consisted of 2 ml S_{25} fraction of rat liver homogenate, 0.2 ml laurate stock solution (final conc. of 0.5 mM, 10^6 dpm), 0.4 ml NADPH generating system, and when needed, 3 mg nicotinamide N-oxide in 0.2 ml buffer. If an anaerobic incubation was needed, thunberg tubes were used with the liver homogenate and the NADPH in the bottom compartment and with the nicotinamide N-oxide (when used) and laurate in the top compartment. The reaction was started by tipping the tube. The incubations were carried out at 37°C for one hour. The incubation was terminated by the addition of 1 ml of 10% sulfuric acid to every 3 ml incubation mixture. The acidic mixture was extracted 7 times with 5 ml of a 9:1 benzene-ether mixture (98% of the added dpm were extracted). The combined extracts were washed with water two times, dried with sodium sulfate, and evaporated to dryness under reduced pressure. The dried extract was dissolved in 0.1 ml methanol and a portion of this was injected into a gas liquid chromatograph (Varian model 204). The peaks were collected in capillary tubes which were

washed out into scintillation vials and counted. The column for the GC was a 3 ft, 1/4 inch glass column packed with Chromsorb 101 (Johns Manville). The helium flow was 60 ml/min. The injector, column oven, and detector were maintained at 280°C. Lauric acid had a retention time of 3.5 min, and the hydroxy acid had a retention time of 8 min.

Results

The peak corresponding to the hydroxylauric acid (the hydroxylated product being assayed) was determined to be a hydroxy acid by collection of the peak from a preparative run and determined the infrared absorption spectra. The IR showed maxima (cm^{-1}) at 3630, 3510, 3400 (broad), 3000, 2920, 2850, 1710, 1440, 1380, 1340, 1320, 1175, 1125, 1080, and 950. This supports the assignment of the product as a hydroxylauric acid.

Table 1 shows the formation of the hydroxylauric acid under various conditions of incubation. The incubations are as described in the experimental section. It can be seen that under anaerobic conditions very little of the radioactivity added in the lauric acid appears in the hydroxylauric acid peak from the GC of the incubation extracts. There is no increase in the formation of hydroxylauric acid when nicotinamide N-oxide is present.

Conclusion

Again the conclusion that can be drawn is that nicotinamide N-oxide is not an intermediate in the activation of oxygen by microsomal hydroxylase systems. This confirms the same finding using the squalene oxidase system.

Table 1

Effect of Nicotinamide N-Oxide on Laurate Hydroxylase

<u>Tube</u>	<u>Atmosphere</u>	<u>NADPH</u>	<u>Nicotinamide N-oxide</u>	<u>% Hydroxylaurate</u>
I	air	+	---	5
II	air	+	10 mM	5
III	helium	+	10 mM	1.5
IV	helium	-	10 mM	2
V	helium	-	---	1.5

II-5 PARAHYDROXYBENZOATE HYDROXYLASE

P-Hydroxybenzoate Hydroxylase

Introduction

Although nicotinamide was implicated in microsomal hydroxylase systems, it was shown that nicotinamide N-oxide did not act as a discrete intermediate in the activation of oxygen for squalene oxidase and laurate hydroxylase. The transitory formation of nicotinamide N-oxide by the enzyme with immediate transfer of oxygen to substrate would not be detected by the experimental approach used. Possibly, the potentiation of microsomal hydroxylase systems by nicotinamide could be due to some conformation change brought about by the nicotinamide in the membranes of the microsomes. Thus it seemed reasonable to look at purified, soluble hydroxylase systems.

P-hydroxybenzoate (POB) hydroxylase from psudonomas was studied since the soluble enzyme has been purified, its molecular weight determined (93,000), and some of the mechanistic aspects of the hydroxylation reaction defined (B. Hesp, M. Calvin and K. Hosokawa, 1969). The effect of nicotinamide and nicotinamide N-oxide on this enzyme were studied.

Experimental

The POB hydroxylase and the C¹⁴ p-hydroxybenzoate were a gift of Dr. Hosokawa. The preparation of the enzyme is given in the paper by K. Hosokawa and R. Stanier (1966). NADPH was purchased from Mann Biochemicals.

In an experiment where anaerobic conditions were necessary, the procedure used was the one described by B. Hesp et al. (1969). Generally buffer, enzyme, and POB were added to a tube and the

solution was deoxygenated by bubbling with helium. NADPH was then added. At this point nicotinamide N-oxide was added to one tube. The tubes were incubated for two hours at room temperature. The reaction was terminated by heating the samples for ten minutes at 85°C. The precipitated protein was centrifuged and a known volume of the supernatant was spotted on chromatographic paper. The paper used for paper chromatography was the "Ederol" brand made by J. C. Binzer (Germany). The paper was then developed with a mixture of 100 ml of 69% butyric acid, 0.85% NaOH and 10 ml benzene. The r_f values for the POB and the product protocathechulic acid (PCA) were 0.8 and 0.6. The paper was then radioautographed. The spots corresponding to the compounds were cut out, eluted with water, and counted on the liquid scintillation counter.

Results

The effect of nicotinamide on the formation of PCA

Two tubes were incubated at 4°C. Tube A contained 30 mM nicotinamide. Each tube contained 3 ml 0.05 M potassium phosphate buffer (pH 7.5), 0.075 ml 2×10^{-7} M POB hydroxylase, 0.020 ml 10^{-1} M POB (10^5 dpm), and 0.020 ml 10^{-1} M NADPH. The contents are added in the order given. At various time intervals 0.3 ml of each tube is removed, placed in a small centrifuge tube, heated, spun, 0.1 ml is spotted on paper, and POB and PCA determined as described. The results are given in Figure 1. As can be seen, nicotinamide had a slight inhibitory effect on the formation of PCA. This experiment was repeated at room temperature, and nicotinamide had no effect on the formation of PCA. (At the end of two hours 70% of the POB had

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Figure 1. Effect of nicotinamide on the conversion of p-hydroxybenzoate (POB) to protocatechulic acid (PCA) by POB hydroxylase. A: (o) contains 30 mM nicotinamide; B: (□) is the control. See text for details on the incubation.

been converted to PCA with or without nicotinamide present.)

The lack of formation of nicotinamide N-oxide from nicotinamide by POB hydroxylase

Essentially the same experiment was performed as the one for the effect of nicotinamide on PCA formation. In this experiment C^{14} nicotinamide was added (final conc. of 3×10^{-3} M), NADPH was 10^{-3} M. In a second tube POB was added in 10^{-3} M conc. to check if possible the N-oxide formed during oxidation of POB. A third tube was made up as a control which contained only buffer, NADPH, and the nicotinamide. Aliquots were taken at 30 min, 60 min, and 180 min. The reaction was terminated by heating, the tubes were spun, and an aliquot was spotted. The chromatographic procedure was the same as described under the nicotinamide N-oxidase. There was no nicotinamide N-oxide formed.

The lack of oxygen transfer from nicotinamide N-oxide under anaerobic conditions

In this experiment nicotinamide N-oxide was added to the reduced FAD-enzyme-substrate complex in an attempt to replace oxygen by nicotinamide N-oxide. Two tubes were employed in each tube, the procedure was identical except for the addition of nicotinamide N-oxide to one. The other then served as a control. Each tube contained 3 ml buffer, 0.020 ml enzyme (2×10^{-5} M), 0.01 ml 10^{-1} M POB (10^6 dpm), some antifoam C above the liquid line. At this point the tubes were deoxygenated by flushing with extra pure helium. At that point, 1.1 μ moles NADPH was added in 0.04 ml buffer. To one tube was then added 5.5 μ moles nicotinamide N-oxide in 0.030 ml.

After allowing the tubes to stand for three hours at room temp. (all above manipulations were done at ice temp.), the tubes were heated to 85°C for 10 min. 0.1 ml was then spotted on paper and the amount of PCA formed was determined as described in previous experiments. This experiment was repeated two times. In each case there was no difference between the control and the experimental tubes containing the N-oxide. (13% PCA formed in each case. This must be due to some slight oxygen leakage or incomplete deoxygenation.)

Conclusion

Neither nicotinamide nor nicotinamide-N-oxide function as co-factors in parahydroxybenzoate hydroxylase.

III CONCLUSION

Conclusion

A model system for microsomal hydroxylation reactions has been developed. This model system, consisting of a photochemically induced oxygen transfer from pyridine N-oxide to a substrate, has proved to be the best model system to date for enzymatic hydroxylation.

An attempt was made to replace oxygen by nicotinamide N-oxide in microsomal hydroxylase systems and in a microbial hydroxylase. This attempt failed. However, the fact that aromatic N-oxides can form some active intermediate which parallels the "activated" molecular oxygen in hydroxylase systems, and the fact that aromatic N-oxides have been shown to transfer oxygen by xanthine oxidase, indicates that aromatic N-oxides may play a role in enzymatic hydroxylations. The possibility that an aromatic N-oxide is produced "in situ" by the enzymatic system, with subsequent oxygen transfer, will have to wait to be studied until the microsomal hydroxylase enzymes have been purified, sequenced, and the active centers determined.

Some of the aspects of these hydroxylase systems which must be kept in mind in further studies are: 1) The enzyme systems must be further purified and characterized; 2) there may be several distinct enzyme systems involved in hydroxylating different substrates; 3) there may be several different mechanisms of action by the same enzyme system for the various substrates. There may be different means of activating molecular oxygen (for example, one electron and two electron transfer centers in the enzyme system) or different cofactor requirements. There is much work being done on these

hydroxylase systems, and some of these aspects of the problem are being defined.

In Figure 1 is proposed a possible mechanism for the activation of O_2 consistent with Bobst's (1968) proposals, using a one-electron transfer center. The scheme shows a generalized aromatic N compound as essential in producing the hydroxylating species (O_2^{2-}). The N involved could be part of a cofactor molecule (such as FAD, pteridine, nicotinamide) or part of a molecule incorporated in the primary structure of the enzyme itself.

Figure 2 shows a possible two-electron transfer center involving an aromatic N compound. This scheme is introduced, since R-O-O⁻ species have been proposed as possible hydroxylating intermediates. The existence of an aromatic N-dioxide has been described by Edwards and Gillespie (1966). A N-O-O⁻ species may be a more active hydroxylating species than a C-O-O⁻ species. These two schemes are presented only as possible structures to consider as participating in the hydroxylation reaction.

The possibility that other kinds of N-oxides may act as intermediates in the activation of molecular oxygen by hydroxylase systems is still open. H. Schmidt and M. Moeller (1968) report that dimethyl aniline oxide can replace molecular oxygen in some NADPH-dependent oxidations catalyzed by rat liver microsomes.*

Thus the question of N-oxides and activation of molecular oxygen by hydroxylases is still open, and many more questions have been asked.

*The authors have not published their findings yet. This information is from abstracts of papers given at a meeting. It is impossible to assess whether these results have significance to the questions in this thesis.

Figure 1. Scheme for oxygen activation by hydroxylase
 incorporating an aromatic N compound and a
 one electron transfer center. XBL 707-5293

Fig. 2

Scheme for oxygen activation by hydroxylase incorporating an aromatic N heterocyclic compound and a two electron transfer center.

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