tion prompted reexamination of the −2.2- to −3.8-ppm region of the spectrum of native myoglobin. With 250 computer-averaged traces, a resonance of 110-cps half-maximum width emerged at −3.72 ppm. These results would seem to justify the conclusion that side-chain groups positioned sufficiently near to the porphyrin ring to experience sizable ring current shifts are present in myoglobin and probably hemoglobin as well as in the previously discussed cytochrome *c*. It would appear that such resonances of high-spin myoglobin and hemoglobin are broadened because of dipolar relaxation by the heme iron, although the effect alternatively could originate in differences in electronic relaxation times of iron in the reduced and oxidized forms of the protein.

Acknowledgments. We are grateful to Dr. R. C. Ferguson for advice and assistance in the maintenance and operation of the 220-Mcps spectrometer and to Messrs. F. W. Barney and F. V. Ferrari for technical assistance in obtaining the spectra.

The Metabolic Products of Naphthalene in Mammalian Systems[1]

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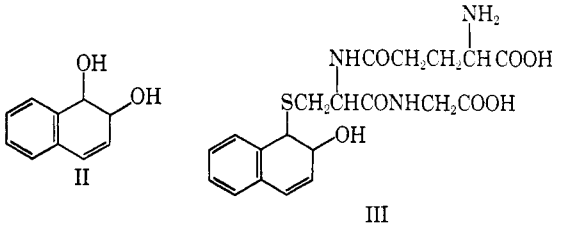
Abstract: The enzymatic conversion of naphthalene to 1,2-dihydro-1,2-dihydroxynaphthalene has been shown by labeling experiments to proceed with the incorporation of one atom of oxygen from molecular oxygen, the second oxygen atom being derived from water. The initial attack upon the substrate takes place at the α position and the product has been shown by nmr spectroscopy to be the *trans* diequatorial diol. The over-all mechanism of the oxygenation is discussed in the light of these findings.

In 1955 it was demonstrated independently by Hayaishi, *et al.*,[2] and by Mason, *et al.*,[3] that molecular oxygen may be directly incorporated into various substrates. Such reactions are now generally termed oxygenations to distinguish them from oxidations, in which molecular oxygen is merely an electron acceptor. It is now quite clear that oxygenases, the enzymes that catalyze oxygenations, are of great importance in that they are involved in the metabolism of a great variety of substrates such as steroids,[4] aromatic hydrocarbons,[5] alkaloids,[6] and numerous drugs[7] such as aminopyrine[8] and the barbiturate, seconal.[9] The metabolism of naphthalene in mammalian systems[10] has been thought[11] to proceed *via* an oxygenation step, the mechanism of which is, however, obscure. As this substrate is representative of the polycyclic carcinogens, it was deemed of interest to study the pathway by which it is oxidatively metabolized.

Among the major metabolites of naphthalene in rabbits and rats are α-naphthol (I), 1,2-dihydro-1,2-dihydroxynaphthalene (DHN-diol, II),[12,13] and S-(1,2-dihydro-2-hydroxy-1-naphthyl)glutathione (III). Further work on *in vitro* systems showed that formation of I and II from naphthalene is catalyzed by liver microsomal enzymes which require NADPH and molecular oxygen.[14] It has not been possible to classify these enzymes as oxygenases since it has not been demonstrated that molecular oxygen is directly incorporated into naphthalene. A molecule of oxygen may be incorporated intact, the enzyme involved being a dioxygenase, or it may donate one atom of oxygen, the enzyme catalyzing such a reaction being a monooxygenase of the mixed function type,[15] since NADPH is required. It is known[14,16] that neither I nor II may serve as a precursor of the other, but whether they both have a common precursor is unclear.



We have already adduced evidence[1] showing that only one atom of molecular oxygen is incorporated into naphthalene in the formation of II and now wish to report experiments which show that naphthalene is converted by a monooxygenase to both I and II, possibly *via* a common precursor.

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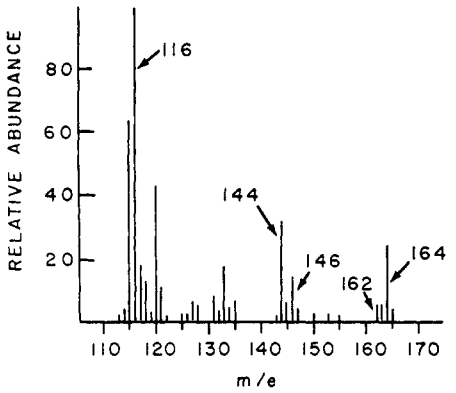
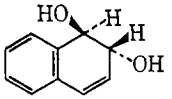


Figure 1. Partial mass spectrum of DHN-diol prepared in an atmosphere containing 77% $^{18}O_2$ and 23% $^{16}O_2$.

A solution of naphthalene in ethylene glycol monomethyl ether was incubated at 37° for 30 min with a mouse liver homogenate preparation in air. The mixture was then made alkaline with sodium carbonate solution, and the products were extracted with ether. The DHN-diol obtained in this way had mp 119–121° and $[\alpha]^{25}D$ +412° (*c* 0.66,$CHCl_3$); *cf.* values quoted by Boyland[10] (mp 125°, $[\alpha]^{25}D$ +159° (*c* 1.00, EtOH)). The 100-Mc nmr spectrum[17] of this diol showed a complex pattern in the aromatic region from 690 to 770 cps[18] and, in addition, doublets at 641 and 594 cps (J = 9.9 cps) and at 483 and 447 cps (J = 10.1 cps), each doublet corresponding to one proton. The downfield pair of doublets clearly correspond to the olefinic protons H_3 and H_4. The coupling constant between these protons is in agreement with values established[19] for such vicinal systems. None of the four doublets appears to be further split, and it may therefore be concluded that coupling between H_2 and H_3 is less than 1 cps. The two doublets at higher field are from protons H_1 and H_2. The high coupling constant between these two protons, when considered in terms of the Karplus relationship,[20] suggests that the dihedral angle between them must be close to either 0° or, more probably, 180°. The very weak coupling between H_2 and H_3 is consistent[21] with a dihedral angle between these two protons of about 90°. Of the four possible conformations of II only the enantiomers IV, in which both the hydroxyl groups are quasiequatorial and *trans* to each other, accommodate these results.



IV

Labeling experiments were undertaken to determine the origin of the oxygen in the α-naphthol and the DHN-diol. It must be decided whether both oxygen atoms in the DHN-diol are from atmospheric oxygen and, if so, whether they are from the same molecule of atmospheric oxygen. To this end, a mixture was prepared containing 80% nitrogen and 20% oxygen which was $^{18}O_2$ (98% by analysis) diluted with a known amount of $^{16}O_2$. This atmosphere was essentially devoid of molecules of ^{18}O–^{16}O. The incubation was then carried out in this atmosphere and the metabolites were isolated in the usual manner.

The mass spectrum of the α-naphthol so obtained showed molecular ions at *m/e* 146 ($C_{10}H_8{}^{18}O$) and 144 ($C_{10}H_8{}^{16}O$) both of which collapsed to give an ion at *m/e* 116 ($C_9H_8^+$) together with the appropriate metastable ions at *m/e* 92.3 and 93.3 (calculated, *m/e* 92.2 and 93.5). The relative abundances of the molecular ion are within 3% of the proportion of $^{18}O_2$ to $^{16}O_2$ used in the incubation.

The purified DHN-diol, prepared in the presence of $^{18}O_2$, had molecular ions at *m/e* 162 ($C_{10}H_{10}{}^{16}O_2$) and 164 ($C_{10}H_{10}{}^{16}O^{18}O$), but not at *m/e* 166 ($C_{10}H_{10}{}^{18}O_2$), showing that only one atom of molecular oxygen is incorporated. The partial high-resolution mass spectrum of the labeled DHN-diol, shown in Table I, confirms the above assignments of formulas. Again the abundance of the ion at *m/e* 164 relative to that at *m/e* 162 paralleled the relative amount of $^{18}O_2$ used in the incubation. Figure 1 shows the molecular ion region of the mass spectrum of DHN-diol, prepared in atmospheres containing 77% $^{18}O_2$ and 23% $^{16}O_2$. Predictably, the most important fragmentation pathway of the DHN-diol molecular ion involves loss of water to give either an α-naphthol ion or a β-naphthol ion. Since the diol can be sublimed unchanged at 200°, the elimination is a result of electron bombardment as opposed to pyrolysis. However, the appearance of ions of both *m/e* 144 ($C_{10}H_8{}^{16}O$) and 146 ($C_{10}H_8{}^{18}O$) would suggest that the elimination of water is only partially specific. It is not possible therefore to determine the position of the ^{18}O in the labeled diol, or even to show that it is not randomized.

Table I. Accurate Mass-to-Charge Ratios in the Mass Spectrum of ^{18}O 1,2-Dihydronaphthalene-1,2-diol

Formula	*m/e* Calcd	*m/e* Found
$C_{10}H_{10}{}^{16}O_2$	162.0687	162.0685
$C_{10}H_{10}{}^{16}O^{18}O$	164.0723	164.0726

Upon treatment with aqueous hydrochloric acid, DHN-diol is smoothly dehydrated to give a mixture of α- and β-naphthols. These may be easily separated by glpc on nonpolar phases such as OV-1, OV-17, or SE-30, and in this way it may be shown that the mixture contains about 95% α-naphthol and 5% β-naphthol. Treatment of the mixture with bis(trimethylsilyl)acetamide[22] gave a mixture of trimethylsilyl ethers which were separated by glpc using OV-1 as the stationary phase. The effluent from the gas chromatograph was depleted in carrier gas by passage through a Ryhage molecular jet separator[23] and then admitted directly into the source of a mass spectrometer. The total ion monitor is used as a gas chromatography detector, its output being shown in Figure 2. Mass spectra MS-1

(17) We are very grateful to Mr. Ernest Gooden, U. S. Department of Agriculture, Beltsville, Md., for his expert assistance in the measurement of this spectrum.

(18) All frequencies are given in cps relative to an internal standard of TMS at 0 cps.

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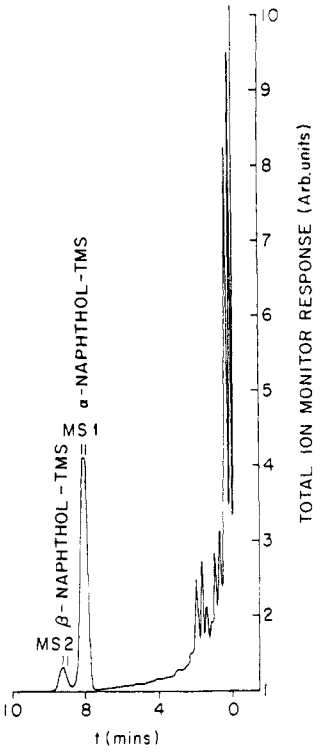


Figure 2. Separation of trimethylsilyl ethers of α- and β-naphthol. The total ion monitor of the mass spectrometer is used as a detector and mass spectra of the effluent peaks are run between the time points shown.

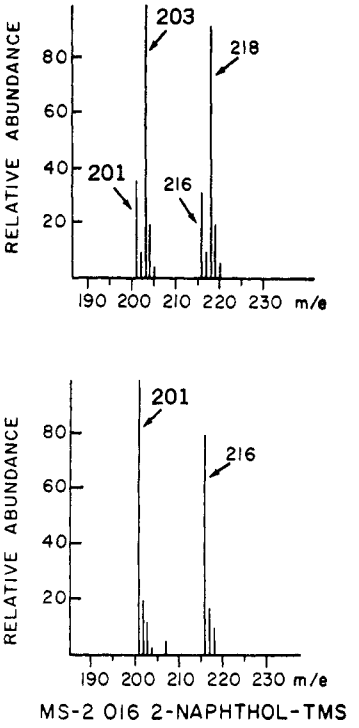
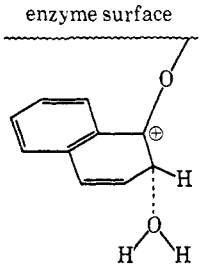


Figure 3. Partial mass spectra of the trimethylsilyl ethers of α- and β-naphthol. The $^{18}O_2$ enrichment used in the incubation is 77%.

and MS-2, each run in 10 sec, were recorded at the time points shown in Figure 2. The molecular ion regions of these two spectra are shown in Figure 3 and reveal quite clearly that, while the trimethylsilyl ether of α-naphthol has molecular ions at *m/e* 218 ($C_{13}H_{16}{}^{18}OSi$) and 216 ($C_{13}H_{16}{}^{16}OSi$) in the expected proportion, the β-naphthol trimethylsilyl ether has no ion at *m/e* 218 other than the usual ^{30}Si isotope. Otherwise, the spectra are uninformative, both showing only the the loss of $\cdot CH_3$, a well established[24] fragmentation of trimethylsilyl ethers. Thus in the labeled DHN-diol, the ^{18}O is all at the α position.

These results imply that the oxygen molecule interacts with the enzymes, and that as a result the oxygen atoms are separated, one subsequently being activated and the other presumably reduced by NADPH. Alternatively, the oxygen molecule may be first activated, then cleaved, but in either case the over-all result is that one oxygen atom is incorporated into the substrate and the other into water. The second oxygen in the diol presumably is incorporated from water, but on a simple statistical basis, the possibility of ^{18}O being incorporated in the diol *via* H_2O can be calculated to be somewhat less than 1% at best. The enzyme responsible can therefore be classified as a mixed function oxygenase within the terms of Mason's definition.[15]

The nature of the activated oxygen is still unclear, but with the data now available, a number of pertinent remarks might be made. The fact that the DHN-diol formed is exclusively the *trans* isomer is consistent with the view that the planar substrate is held on the surface of the enzyme which introduces the first oxygen. The intermediate so formed, which may be represented as, for example, V, must acquire the second oxygen from the attack of water on the opposite face of the substrate to the first oxygen to form the *trans* diol. Alternatively, loss of H^+ from carbon 1 of V will give α-naphthol, which is subsequently released from the enzyme surface.



V

Simple rearrangement of V could lead to naphthalene 1,2-epoxide which has been postulated[13] as a metabolite of naphthalene in mammalian systems since upon reaction with glutathione it might be expected to lead to the mercapturic acid III.

The formation of α-naphthol and the position of the ^{18}O in the DHN-diol both suggest that the initial attack upon naphthalene takes place at the α position. While it is tempting to infer from this that the oxygenating species is an electrophile, such as OH^+, which would be expected to attack at this position of higher electron density,[25] this is to ignore the possibility that it is the enzyme itself that confers specificity upon the reaction. Thus radical species such as $\cdot OH$, gen-

(24) A. G. Sharkey, R. A. Friedel, and S. H. Langer, *Anal. Chem.*, 29, 770 (1957).

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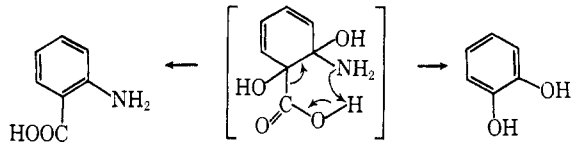
erated[26] by the Udenfriend reagent[27] or $O_2H\cdot$,[28] which are generally rather nonspecific,[29] cannot be ruled out, any more than can the "oxenoid" species postulated by Hamilton.[30]

A quite different mechanism of oxygenation apparently occurs in microorganisms. A dioxygenase isolated[31] from *Pseudomonas* effects the incorporation of oxygen into anthranilic acid giving presumably the dihydro diol VI which spontaneously loses CO_2 and NH_3, to give catechol, possibly as shown. It has been suggested[32] on the basis of ^{18}O-labeling experiments that both atoms originate from molecular oxygen and that the cyclic peroxide VII is an intermediate in the formation of VI. The ring junction in VII must be *cis* and it follows therefore that the carboxyl group



VI

and the amino group in VI must also be *cis* to one another, thus facilitating the cyclic elimination shown.

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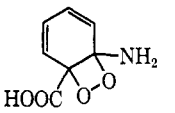

VII

Experimental Section

Gas–liquid partition chromatography was carried out on a Glowall Model 310 chromatograph equipped with a 10-Mc ^{90}Sr argon ionization detector at 1000 v, using argon as a carrier gas. Mass spectra were measured on an A.E.I. MS-9 double-focussing mass spectrometer at 70 ev and on an LKB 9000 gas chromatograph–mass spectrometer, also at 70 ev. Accurate mass measurements were made by peak matching using perfluorotributylamine as a standard. Nuclear magnetic resonance spectra were measured in a Varian Associates HA-100 spectrometer using a frequency sweep.

The preparation of the mouse liver homogenate supernatant, the determination of its enzymatic activity, and the conversion of naphthalene to DHN-diol by this preparation have already been described.[1] The DHN-diol was purified by vacuum sublimation and recrystallization from benzene as colorless plates, mp 119–121°.

Enzymatic Formation of α-Naphthol. The α-naphthol formed in the enzymatic reaction was separated from the DHN-diol by thin layer chromatography on silica gel, and purified by glpc as its trimethylsilyl ether.

Nonenzymatic Formation of α-Naphthol. DHN-diol (*ca.* 0.1 mg) was dissolved in 2 *N* HCl (0.1 ml), and the solution was heated at 100° for 20 min. The aqueous solution was then extracted with ether (two 10-ml portions), and the ether was then evaporated off to give a mixture of α- and β-naphthols which were separated by glpc as above.

Preparation and Glpc of Trimethylsilyl Ethers. The mixture of naphthols obtained from 0.1 mg of DHN-diol was dissolved in bis(trimethylsilyl)acetamide (1 ml), and the mixture was allowed to stand at room temperature for 1 min, then chromatographed on (typically) a 6-ft column of 3% OV-1[33] at 120° with a carrier gas (He) pressure of 25 psi.

(33) Applied Science Laboratories, Inc., State College, Pa.

Molecular Sieve Entrapment. I. Entrapment of Deoxyribonucleic Acid by Cross-Linked Dextran (Sephadex)[1]

Robert Yolles

Contribution from Stanford Research Institute, Menlo Park, California 94025.
Received July 7, 1967

Abstract: Mechanical trapping of deoxyribonucleic acid (DNA) in a three-dimensional, hydrophilic matrix was accomplished by cross-linking dextran in the presence of single-stranded DNA. Diffusion of the trapped DNA molecules out of the three-dimensional matrix depends on the extent of cross-linking of the resin and on the time at which the DNA is added after initiation of the cross-linking reaction. Reactions of the resin-trapped DNA with nucleases of different molecular weights show that the ability of a nuclease to attack the trapped DNA depends on the size of the enzyme and on the degree of cross-linking of the resin.

Although clathrates or inclusion compounds are well known for small molecules,[2] and the diffusion of large and small molecules into molecular sieves has been amply described,[3] there is a scarcity of reports on the entrapment of large molecules into molecular sieves. This work deals with the *in situ* entrapment of macromolecules by molecular sieves. The feasibility of molecular sieve entrapment in a hydrophilic system was tested by trapping deoxyribonucleic acid (DNA) into Sephadex (a cross-linked dextran) by allowing the cross-linking reaction to take place in the presence of DNA, a macromolecule found suitable for this work because of its high molecular weight and well-characterized chemical properties.

(1) The author wishes to thank Dr. G. Freeman for consultive support, Mr. R. J. Jones for excellent technical assistance, and Dr. Z. Reyes for reading the manuscript prior to publication.
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