A Mass Spectrometric Investigation of Pyridoxol, Its C-5 Analogs, and O-Isopropylidene Derivatives[1,2]

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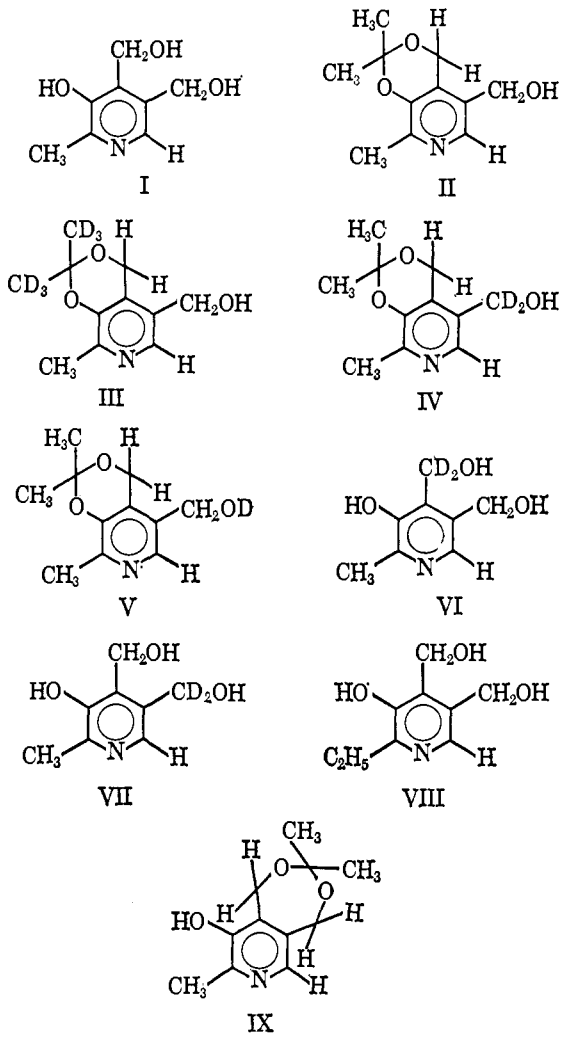
Abstract: The mass spectra of pyridoxol, its two isomeric isopropylidene derivatives, several 5-modified pyridoxol analogs, and the corresponding deuterated compounds have been obtained. Molecular ions were observed for all compounds. Fragmentation patterns characteristic of the nature and position of substituents were interpreted in each case with the aid of metastable peaks, deuterium labeling, and analogies with similar systems. Mass spectra of these compounds can serve as models for the determination of structures of metabolites and analogs of pyridoxol in connection with chemical and biological investigations in this area.

Pyridoxol (I), pyridoxal, pyridoxamine, and their phosphates comprise a group of substances designated as vitamin B_6. The central role of this vitamin in nitrogen metabolism has resulted in studies directed toward the synthesis of metabolites, potential antagonists, and model systems. Also, a number of new metabolites of this vitamin have been isolated in recent years.

Although the various forms of vitamin B_6 are widely distributed in nature, their concentrations in various microorganisms and tissues are very low, and hence much effort has been devoted to the development of analytical methods.[3] Among the methods used in vitamin B_6 chemistry, ultraviolet,[4] infrared,[5] and nuclear magnetic resonance[6] spectroscopy have proved useful, especially for structure elucidation.

The small amount of sample required for mass spectral analysis and the success of mass spectrometry in structural studies of natural products suggest that mass spectrometry might find application in the field of vitamin B_6 chemistry.[7]

This paper reports the electron-impact fragmentation of pyridoxol (I), its α^4,3-O-isopropylidene derivative (II), a number of deuterated analogs (III–VII), and ω-methylpyridoxol (VIII). In addition, the mass spectra of α^4,α^5-isopropylidenepyridoxol (IX), which is a second isopropylidene derivative of pyridoxol, and of some synthetic vitamin B_6 compounds modified at the 5 position will be discussed.



(1) Pyridoxine Chemistry. X. For paper IX see W. Korytnyk and B. Paul, *J. Heterocyclic Chem.*, **2**, 481 (1965).

(2) (a) Presented in part at the 13th Annual Conference on Mass Spectrometry and Allied Topics, St. Louis, Mo., May 1965. (b) This investigation was supported in part by the National Institutes of Health, U. S. Public Health Service (Grant CA-05697); the mass spectrometer was purchased by Wayne State University with a Research Instruments Grant from the National Science Foundation.

(3) (a) C. A. Storvick and J. M. Peters, *Vitamins Hormones*, **22**, 833 (1964); (b) C. A. Storvick, E. M. Benson, M. A. Edwards, and M. J. Woodring, *Methods Biochem. Analy.*, **12**, 183 (1964); (c) E. W. Toepfer and M. M. Polansky, *Vitamins Hormones*, **22**, 825 (1964).

(4) D. E. Metzler and E. E. Snell, *J. Am. Chem. Soc.*, 77, 2431 (1955).

(5) F. J. Anderson and A. E. Martell, *ibid.*, **86**, 715 (1964).

(6) W. Korytnyk and R. P. Singh, *ibid.*, **85**, 2813 (1963), and ref 1.

(7) For a review, see (a) K. Biemann, "Mass Spectrometry," McGraw-Hill Book Co., Inc., New York, N.Y., 1962; (b) H. Budzikiewicz, C. Djerassi, and D. H. Williams, "Interpretation of Mass Spectra of Organic Compounds," Holden-Day, San Francisco, Calif., 1964; (c) H. Budzikiewicz, C. Djerassi, and D. H. Williams, "Structure Elucidation of Natural Products by Mass Spectrometry," Vol. 1 and 2, Holden-Day, Inc., San Francisco, Calif., 1964.

Results and Discussion

Pyridoxol and α^4,3-O-Isopropylidenepyridoxol. The mass spectra in Figures 1–3 are of compounds I, II, and IV, respectively. The four peaks of intensity >40% of the base peak in the mass spectra of compounds I, II, and VIII are given in Table I along with their locations in the mass spectra of deuterated analogs III–VII.

Except for the location of the molecular ion peak, the mass spectra of compounds I (Figure 1), II (Figure 2),

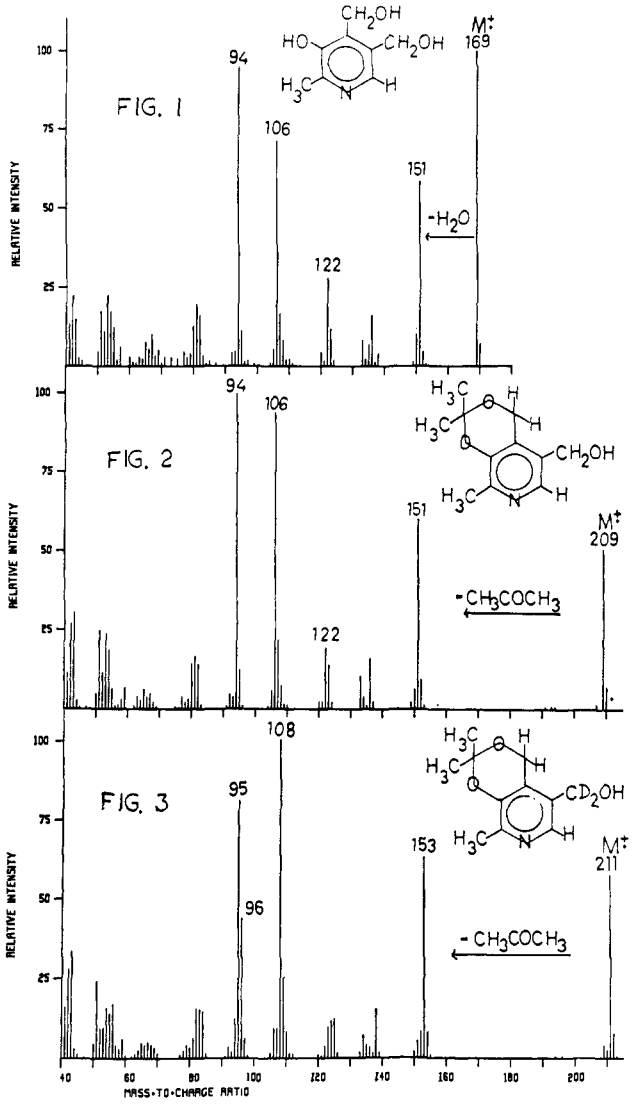
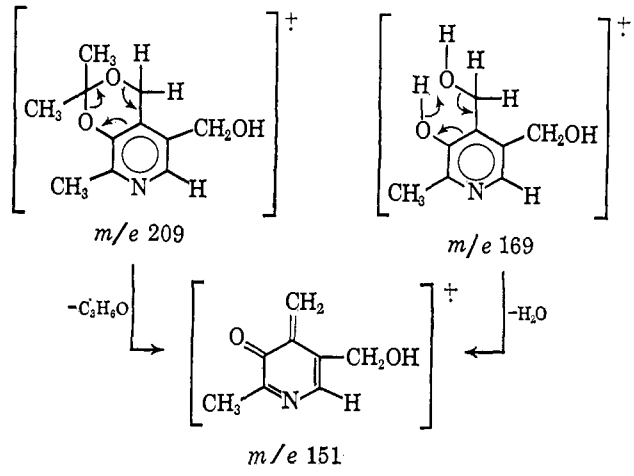
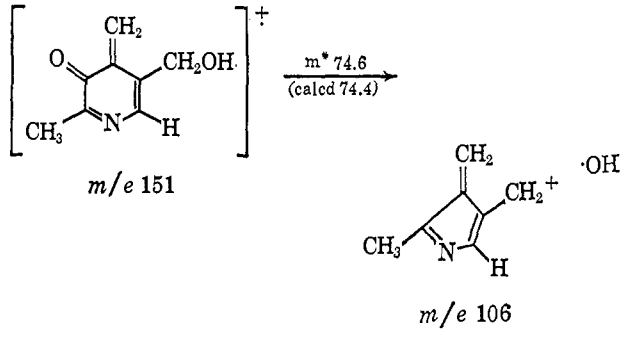


Figure 1. The mass spectrum of pyridoxol.
Figure 2. The mass spectrum of α^4,3-O-isopropylidenepyridoxol.
Figure 3. The mass spectrum of α^4,3-O-isopropylidene-α^5-d_2-pyridoxol.

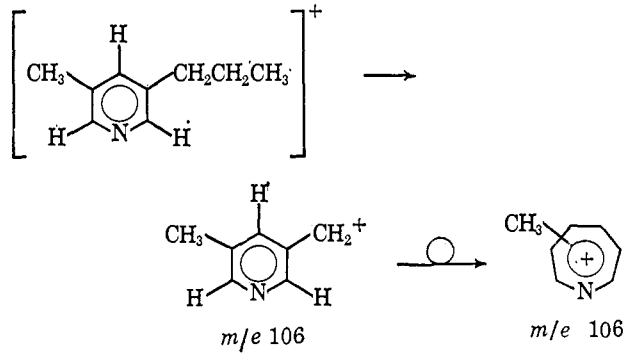
and III show a large degree of similarity. This can be rationalized by the formation of an identical ion at *m/e* 151 from which all further fragmentation occurs. The data in Table I from compounds I–VIII and metastable ion peaks are consistent with the formation of an orthoquinoid structure resulting from the elimination of either water or acetone from the molecular ion. Shannon has proposed a similar orthoquinoid structure for the loss of water from the molecular ion of *o*-hydroxybenzyl alcohol.[8]



The data in Table I and metastable ion peaks indicate that the fragment at *m/e* 151 (Figures 1 and 2) loses CO and HO· to give a fragment at *m/e* 106. The



structure shown for the fragment at *m/e* 106 should be used only to visualize what part of the original molecule remains. It is possible that this 106 fragment undergoes ring expansion to the same azatropylium structure as suggested for the fragmentation of 3-methyl-5-*n*-propylpyridine.[9] Expulsion of carbon mon-



oxide from an orthoquinoid structure has been suggested in the further fragmentation of the M − H_2O ion from *o*-hydroxybenzyl alcohol.[8]

The metastable ion peak for a double fragmentation is unusual. It shifts correspondingly in the mass spectra of compounds I–VIII. It is possible that the major fragmentation is stepwise, but that a fraction of

Table I. Major Peaks in the Mass Spectra of Compounds I–VIII

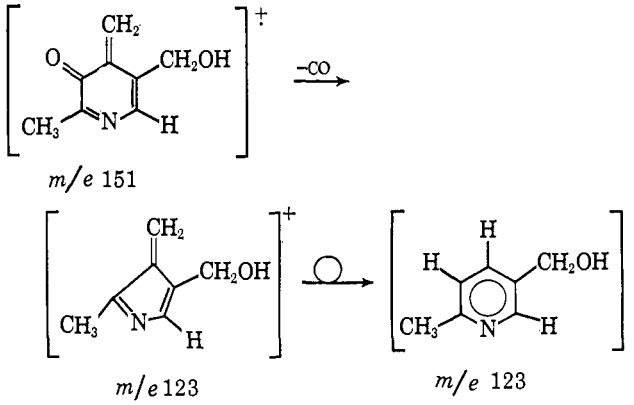
Compd	*m/e*			
I	169 (M^+)	151	106	94
II	209 (M^+)	151	106	94
III	215 (M^+)	151	106	94
IV	211 (M^+)	153	108	95, 96 (2:1)[a]
V	210 (M^+)	152	106	95
VI	171 (M^+)	153	108	95, 96 (1:5)[a]
VII	171 (M^+)	153	108	95, 96 (2:1)[a]
VIII[b]	183 (M^+)	165	120	108

[a] The numbers in parentheses are the intensities of the peaks relative to one another. [b] Also of intensity >40% of the base peak are *m/e* 164, 137, and 136.

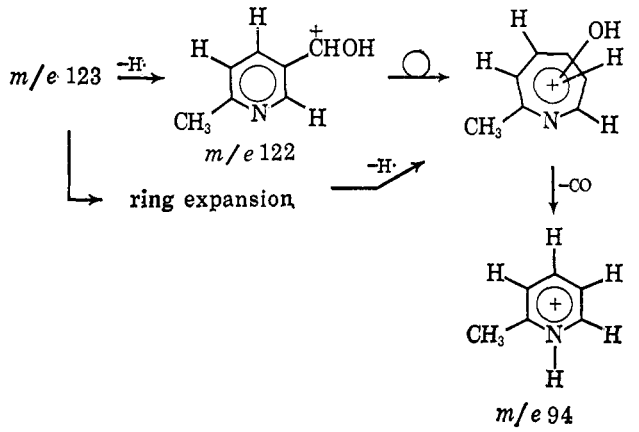
(8) J. S. Shannon, *Australian J. Chem.*, **15**, 265 (1962).
(9) Reference 7b, 255.

the ions fragment in one step. An alternate possibility is that at least a fraction of the ions at *m/e* 151 drastically rearranges to a structure that loses $\cdot CO_2H$ in a single step.

The formation of the base peak at *m/e* 94 in Figure 1 can be also rationalized as arising from the *o*-quinoid ion, *m/e* 151. Elimination of carbon monoxide from fragment 151 and ring expansion would lead to a fragment at *m/e* 123 which conceivably could have the same structure as the molecular ion from a benzyl alcohol analog. If fragment 123 is indeed similar to



the molecular ion from benzyl alcohol, it would be expected also to lose one hydrogen atom followed by carbon monoxide;[8] a metastable ion peak shows that *m/e* 122 is formed from *m/e* 123. Loss of carbon monoxide from *m/e* 122 leads to the base peak at *m/e* 94. The mass spectrum of compound VIII shows these peaks 14 mass units higher and has metastable ion peaks for all the fragmentations, including *m/e* 136 → *m/e* 108. The mass spectra of compounds IV, VI, and VII (Table I) show that loss of a benzylic hydrogen atom as shown above does not explain the loss of deuterium from the hydroxymethylene groups at C-4 and C-5 in the formation of *m/e* 94; there appears to be either a competing loss of hydrogen from other parts of the molecule or some randomization of hydrogen atoms in the formation of *m/e* 122. By using deuterium labeling, Shannon[8] has found that the M − 1 species from the molecular ion of benzyl alcohol owes its genesis to random loss of a hydrogen atom from all carbon atoms; he suggests a π-complex intermediate radical cation to account for this, with ring expansion from benzyl to tropylium occurring before M − 1 is formed. The data in Table I on the shift of *m/e* 94 to *m/e* 95 and 96 for compounds IV, VI, and VII also can be rationalized



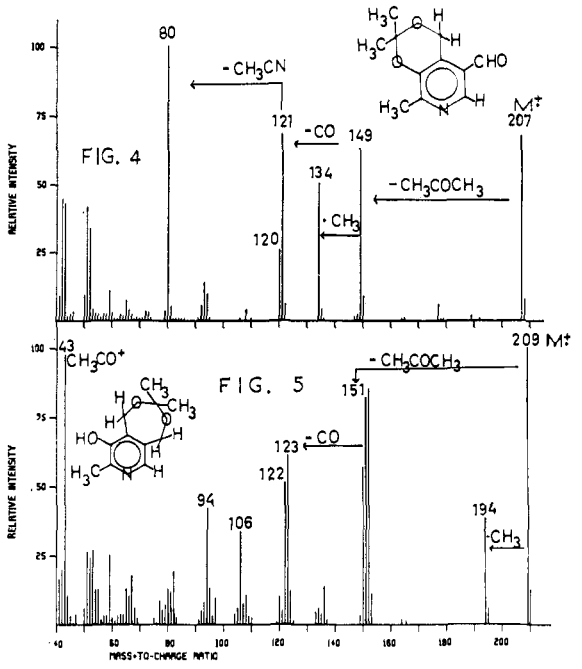
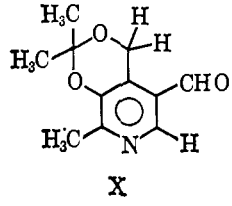


Figure 4. The mass spectrum of α^4,3-O-isopropylidenepyridoxol.
Figure 5. The mass spectrum of α^4,α^5-isopropylidenepyridoxol.

by a competing ring expansion of the fragment at *m/e* 123 to tropylium structure before *m/e* 122 is formed.

The structures proposed for the ions found in the mass spectra are speculative. Nevertheless, the similarities of behavior between pyridoxol and *o*-hydroxybenzyl alcohol, and pyridoxol and benzyl alcohol, on electron impact, are real. With regard to electron-impact fragmentations, the mass spectra of these vitamin B_6 compounds and derivatives are particularly interesting because they can be interpreted in terms of the interplay of the several functional groups present in the molecules.

5-Modified Pyridoxol Analogs. A very striking change in the fragmentation pattern of vitamin B_6 compounds is observed if the hydroxymethylene group on C-5 is replaced with an aldehyde group. The mass spectrum of α^4,3-O-isopropylideneisopyridoxal (X) is shown in Figure 4. Metastable ion peaks are present for the loss of acetone from the molecular ion to give *m/e* 149, and for the loss of a methyl radical and the loss of carbon monoxide from *m/e* 149.

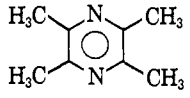


X

Cleavage of a bond at C-5 β to the ring is no longer the predominant fragmentation. Instead, compound X fragments by expelling CH_3CN from the ring. This type of fragmentation is characteristic of pyridine, the only abundant fragment ion of which corresponds to loss of HCN.[10] The mass spectrum of tetramethyl-

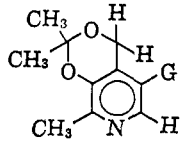
(10) "Catalog of Mass Spectra Data," American Petroleum Institute Research Project 44, Carnegie Institute of Technology, Pittsburgh, Pa., Spectrum No. 617.

pyrazine (XI) shows small peaks for the loss of a methyl radical from the molecular ion and for the loss of CH_3CN from the molecular ion, and a base peak corresponding to the loss of two molecules of CH_3CN from the molecular ion.[11] Table II indicates that other 5-modified pyridoxol analogs without the hydroxymethylene group at C-5 undergo fragmentation analogous to that of compound X.



XI

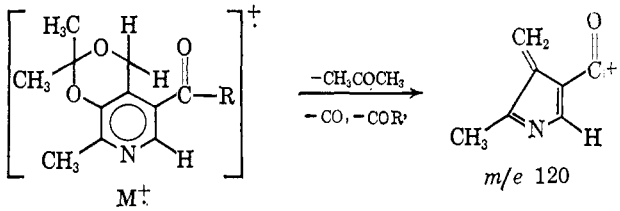
Table II. Mass Spectral Data with G Varied



G (mass of G)	M − 58 − 15 (relative abundance), *m/e* (%)	M − 58 − 28 − 41 (relative abundance), *m/e* (%)
1, CHO (29)	134 (50)	80 (100)
2, $COCH_3$ (43)	148 (60)	94 (42)
3, $CONH_2$ (44)	149 (40)	95 (19)
4, COOH (45)	150 (100)	96 (47)
5, CO_2CH_3 (59)	164 (100)	110 (41)
6, NH_2[a]	...	67 (22)[b]

[a] The base peak in the mass spectrum of this compound results from loss of acetone from the molecular ion. [b] The relative abundance of M − 58 − 28 is 80%.

The fact that cleavage of a bond β to the ring at C-5 does occur in some of these compounds is indicated in the mass spectra of compound X and of the compounds in Table II with G corresponding to $COCH_3$, $CONH_2$, COOH, and CO_2CH_3. This leads to *m/e* 120 in all five examples. The structure shown below visualizes what part of the molecule is present in this fragment; it is likely that the actual structure of the ion involves ring expansion. The relative intensities of *m/e* 120 range from 60% for the methyl ester (R = OCH_3) to 8% for the acid (R = OH).

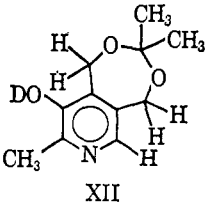


M^+ *m/e* 120

α^4,α^5-Isopropylidenepyridoxol. The mass spectra of the seven-membered ring ketal derivative of pyridoxol (IX, Figure 5), isomeric with compound II, and of a deuterated analog XII, show similarities to and differences from the corresponding mass spectra of compounds II and V. The fragmentation of compound IX is analogous to that of the five-membered ring ketals formed from carbohydrates;[12] a molecule of acetone is lost from the isopropylidene ring, or a methyl radical, followed by ketene, from the acetoxyl group. A

ring expansion of *m/e* 194 to an azatropylium structure is possible.

The peaks at *m/e* 122 and 123 result from expulsion of carbon monoxide from *m/e* 150 and 151, respectively, an observation supported by appropriate metastable ion peaks. Fragments at *m/e* 94 and *m/e* 106 are much less intense than in the mass spectrum of compound II.



XII

Conclusions

Mass spectrometry provides a new method for the structural analysis and identification of pyridoxol and its derivatives, its suitability for identification on a small scale making it useful in many chemical and biological studies in this field. In studies of the biosynthesis of vitamin B_6, the characteristic mass spectra can be used to establish the position and quantitative analysis of stable isotopes, *e.g.*, N^{15} and D, without resorting to complex degradation procedures. The differences in the mass spectra of the two isomeric O-isopropylidenepyridoxols and the variations in the fragmentation patterns as the substituent on C-5 is varied demonstrate how structural features can be recognized from the mass spectra. The mass spectra of pyridoxol and its derivatives are especially interesting in relation to electron-impact fragmentation mechanisms, since these compounds can be viewed from different standpoints as substituted pyridine, benzaldehyde, benzyl alcohol, *o*- and *m*-hydroxybenzyl alcohols and their O-isopropylidene derivatives, and phenol.

Experimental Section

Mass Spectra. The mass spectra were determined with an Atlas CH4 mass spectrometer, ionizing potential 70 ev, ionizing current 18 μa. The solid samples were ionized by electron bombardment after sublimation directly into the electron beam from a small graphite crucible heated by a tungsten coil. A cathode with a tungsten wire of 0.15-mm diameter was used.

(11) Reference 10, spectrum No. 1350.

(12) Don C. DeJongh and K. Biemann, *J. Am. Chem. Soc.*, **86**, 67 (1964).

Above *m/e* 40 the mass spectra are identical whether the compounds are introduced into the mass spectrometer as hydrochlorides or free bases, indicating dissociation before electron bombardment.

α^4,3-O-Isopropylidene-d_6-pyridoxol (III). Pyridoxol hydrochloride (72 mg), suspended in acetone-d_6 (6 ml), was cooled in ice, and hydrogen chloride gas was passed in for 45 min. The reaction mixture was shaken for 2 hr at room temperature. The resulting isopropylidene derivative was precipitated with ether, and was washed with ether, mp 210° dec, undepressed by addition of undeuterated material (lit.[13] mp 212°, dec). The hydrochloride was converted to the free base with $NaHCO_3$. The free base was recrystallized from water, mp 112–114° (lit.[13] mp 111–112°).

(13) W. Korytnyk and W. Wiedeman, *J. Chem. Soc.*, 2531 (1962).

Other Compounds. The syntheses of the undeuterated cyclic ketals of pyridoxol (II and IX) have been reported.[13,14] Also, the preparation and characterization of the deuterated compounds IV, VI, and VII have been described in the preceding paper of this series.[1] The chemistry of α^4,3-O-isopropylideneisopyridoxal (X),[15] and of the other compounds in Table II (2,[16] 3–5,[15] 6[17]), has been described. ω-Methylpyridoxol (VIII) hydrochloride was kindly provided by Dr. S. A. Harris of Merck and Co., Inc.

(14) W. Korytnyk, *J. Org. Chem.*, 27, 3724 (1962).
(15) W. Korytnyk, E. J. Kris, and R. P. Singh, *ibid.*, **29**, 574 (1964).
(16) W. Korytnyk, B. Paul, and B. Garrecht, Abstracts, 148th National Meeting of the American Chemical Society, 1964, p 12P.
(17) W. Korytnyk and B. Paul, *J. Heterocyclic Chem.*, **2**, 144 (1965).

Symmetrical Exchange Reactions at an Aromatic Carbon Atom[1]

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Contribution from the School of Chemistry of the Leicester College of Technology, Leicester, England.[2] *Received November 4, 1965*

Abstract: The order of reactivities of aromatic ethers with sodium methoxide is *p*-nitroanisole < 4-methoxypyridine 1-oxide < 2,4-dinitroanisole < 2,4,6-trinitroanisole. 2,4,6-Trinitroanisole (methyl-^{14}C) undergoes "methanolysis" and forms a red complex with sodium methoxide in methanol which is insoluble in toluene. Subsequent decomposition of the complex with dilute acid results in a nearly 50% decrease in the initial activity of the labeled aromatic compound. The rate of methoxyl exchange for this compound is increased with increasing methoxide ion concentration and reaches a maximum when the reactants have an equal concentration. No "methanolysis" or complex formation is observed between sodium methoxide and 2,4-dinitroanisole or 4-methoxypyridine 1-oxide in methanol. The second-order rate coefficient for methoxyl exchange for 2,4-dinitroanisole is independent of methoxide ion concentration. The mechanism of the methoxyl exchange reactions for the compounds studied in this work is discussed in terms of an intermediate complex formation which is fast for 2,4,6-trinitroanisole but rate determining for 2,4-dinitroanisole and 4-methoxypyridine 1-oxide. From the energies of activation and the heat of formation of the 2,4,6-trinitroanisole–sodium methoxide complex potential energy diagrams are drawn to support the proposed mechanism.

The mechanism of bimolecular nucleophilic aromatic substitution usually has been considered to involve an intermediate complex, formed by addition of the nucleophile to the aromatic carbon atom undergoing substitution. Evidence in favor of the two-step mechanism has been summarized.[3–6] The intermediate complex formed between 2,4,6-trinitroanisole and potassium methoxide has been shown to have a covalent structure by Gold and Crampton,[7] who found only a single methyl resonance peak in the nmr spectrum of the complex in acetonitrile with intensity twice that observed for the parent ether in the same solvent.[7]

Symmetrical exchange reactions offer simple systems for the study of nucleophilic aromatic substitution due to the identity of the reagent and of the displaced group. For methoxyl exchange a study of the activating effect of the nitro groups and the hetero N→O (in pyridine 1-oxide) is reported here. Additional evidence is also presented in support of the intermediate complex mechanism and for the covalent structure of the complex.

(1) Preliminary account: J. H. Fendler, *Chem. Ind.* (London), 764, (1965).
(2) Present address: Department of Chemistry, University of California, Santa Barbara, Calif.
(3) J. F. Bunnett and R. E. Zahler, *Chem. Rev.*, **49**, 273 (1951).
(4) J. F. Bunnett, *Quart. Rev.* (London), **12**, 1 (1958).
(5) J. Sauer and J. R. Huisgen, *Angew. Chem.*, **72**, 294 (1960).
(6) S. D. Ross, *Progr. Phys. Org. Chem.*, 1, 31 (1963).
(7) M. R. Crampton and V. Gold, *J. Chem. Soc.*, 4293 (1964).

Experimental Section

Methanol (AnalaR) was treated with iodine and aqueous sodium hydroxide to convert acetone and ethanol into iodoform, which was filtered off. The filtrate was refluxed for several days to remove traces of iodoform, and twice fractionated.[8] The water content was determined by the Karl Fischer method[9] to be 0.13%.

Methanol (carbon-14 labeled) was purchased from U.K.A.E.A. in sealed ampoules. Its specific activity was 50 μcuries/ml.

Reagent grade toluene, ethyl acetate, and hydrochloric acid were used without purification.

All the aromatic ethers studied in this work are stable, and since carbon-14 has a long half-life, it was convenient to label the organic compounds rather than the sodium methoxide.

p-Nitroanisole (methyl-^{14}C) was prepared by adding 4 ml of methanol in small portions to 0.7 g of sodium in a well-cooled flask equipped with a reflux condensor and drying tube. After the reaction had subsided, 2 ml of labeled methanol containing 200 μcuries of carbon-14 and 2 ml of inactive methanol were added. The sodium methoxide solution was refluxed for 1 hr and cooled to room temperature, when 4.2 g of *p*-dinitrobenzene and 1 ml of inactive methanol were added. The reaction mixture was gently heated for 10 min. The almost solid mass was diluted with 50 ml of water, acidified with 2 *N* hydrochloric acid, and made slightly

(8) H. H. Bates, J. M. Mullaly, and J. H. Hartley, *ibid.*, 401 (1923).
(9) I. M. Kolthoff and R. Belcher in "Volumetric Analysis," Vol. 3, Interscience Publishers, Inc., New York, N. Y., 1957, p 409.