Et_2O to give 2.3 g (77%): mp 185°; $[\alpha]^{31}D$ −0.55° (c 0.92, DMF). *Anal.* ($C_{30}H_{36}Cl_6N_4O_8$) C, H, N.

Poly(*O*-Me-Tyr-Glu-Ala-Gly)Gly-*l*-^{14}*C*-OEt (1).—To a soln of 1 mg (7.6 mmoles) of glycine-*l*-^{14}*C*-OEt·HCl (specific activity 3.15 mCi/mmole) and 1.53 g (15.1 mmoles) of Et_3N in 5 ml of DMSO was slowly added a soln of 2.3 g (2.9 mmoles) of the polymerizing unit **4** in 15 ml of DMSO. The transfer vessels were washed with 9.5 ml of DMSO which was added to the reaction mixt giving a final concn of 100 mmoles/l. The reaction mixt was shaken for 6 days and then centrifuged to yield the product which was washed with three 35-ml portions of H_2O and three 35-ml portions of Et_2O and dried to give the blocked polymer. This material was dissolved in 50 ml of 90% F_3CCO_2H and stirred for 1 hr, and then concd under reduced pressure to yield the crude polypeptide **1**. This material was washed with Et_2O, suspended in 20 ml of H_2O, and dissolved by the addition of 1 *N* NaOH to pH 7.8. The soln was dialyzed against distilled H_2O for 12 hr, acidified to pH 2.5 with HCl, and dialyzed for 3 days. The pptd polypeptide was collected by centrifugation and then lyophilized to yield 0.3 g (24%): radioassay, 35.2 nCi/mg indicates 47% incorporation of the radioactive label. *Anal.* ($C_{20}H_{26}N_4O_7 \cdot H_2O$) C, H, N.

Mol Wt Determination.—Calibrated columns of Sephadex G-100 (2.5 × 38.5 cm) and of Corning CPG 10-240 glass granules (2.0 × 28 cm) were employed for the mol wt detn. Using 0.1 *M* NaCl–0.05 *M* KH_2PO_4 corrected to pH 8.0 as eluent, 4.2 mg of poly(*O*-Me-Tyr-Glu-Ala-Gly)Gly-*l*-^{14}*C*-OEt was passed through each of these columns. The polypeptide was eluted from each column in a vol equiv to that corresponding to a mol wt of at least 1 × 10^5.

Immunochemical Results.—Two rabbits were treated at weekly intervals with 500 μg of poly(*O*-Me-Tyr-Glu-Ala-Gly)Gly-*l*-^{14}*C*-OEt 1. The first 2 weeks they were injected intradermally using complete Freunds adjuvant as suspension medium and the 3rd week they were injected sc. The injection on the 4th week was done iv using buffered saline. Bleedings were conducted on the following week and the serum from each animal was found to give a precipitin reaction with the antigenic polypeptide 1. The preimmunized sera under the same conditions gave a negative precipitin reaction. The quantitative determination of the total precipitate was obtained by the addition of dils of poly(*O*-Me-Tyr-Glu-Ala-Gly)Gly-*l*-^{14}*C*-OEt (1) to 2-ml samples of the pooled rabbit sera. The samples were incubated at 37° for 1 hr and then stood at 4° for 48 hr. The ppts were collected by centrifugation, washed twice with buffered saline, and collected. The total amount of protein precipitated was estimated by analysis for N (Kjeldahl). From these results the precipitin curve shown in Figure 1 was obtained.

Acknowledgments.—This work was supported by a grant from the National Science Foundation.

Analogs of Vitamin B_6 with Reactive Groups[1]

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Received November 13, 1970

Pyridoxol analogs that have alkylating groups in appropriate positions may react irreversibly with cofactor sites of appropriate apoenzymes, or, more generally, with any of the receptor sites that are available for the various biologically active forms of vitamin B_6.[2,3] In order to obtain an analog with dimensions comparable to those of the cofactor forms of vitamin B_6 (pyridoxal or pyridoxamine phosphates), we synthesized the chloro ketone V (Scheme I) and have also attempted to obtain the lower homologs of this compd. The latter were considered as potential irreversible inhibitors, primarily for enzymes that have nonphosphorylated forms of vitamin B_6 as substrates.

As the starting materials for the syntheses of the α-chloro ketones, we used the homologous carboxylic acids (I, VI, XIII),[4] which were protected with an α^4,3-*O*-isopropylidene group. The higher homologs (I and VI) could be readily converted to the corresponding acid chlorides by carefully reacting the acids with $SOCl_2$ in C_6H_6, but we could not obtain an acid chloride from the lowest homolog XIII by this procedure. Variations of reaction conditions with different chlorinating reagents did not give the desired result.[5]

Addition of the acid chlorides II and VII to a large excess of CH_2N_2 in Et_2O resulted in the formation of the diazo ketones III and VIII. The higher homolog II also gave an appreciable amt of the α-chloro ketone IV, which was isolated; the mixt of III and IV, however, gave the desired α-chloro ketone V on treatment with HCl, as indicated by a positive Baker's test[6] (a test for active halogen, applied as a tlc spray reagent), and by ir and nmr spectra. The lower homolog VIII, on being similarly treated with HCl, gave a mixt of products, with a negative Baker's test. Treatment of the diazo ketone VIII with ethereal HCl resulted in the formation of the blocked α-chloro ketone IX, as indicated by a positive Baker's tests. Mild hydrolysis of the protecting isopropylidene group gave exactly the same mixt of products as was obtained directly from the diazo ketone VIII by treatment with HCl. The 2 products were separated by preparative tlc, and the main product was shown to be the bicyclic hemiacetal X of the α-chloro ketone, and was further characterized as the diacetyl derivative XII. The minor component of the reaction mixt was probably formed by dehydration of the major compd X, and it was indicated by nmr to be XI (see Experimental Section). The small amt of the sample precluded the further work necessary for making an unequivocal structural assignment.

The α-chloro ketone V was tested as an inhibitor of several enzymes. It did not inhibit apotryptophanase[7,8] at 5 × 10^{-4} *M*; but when the concn was raised to 4 × 10^{-2} *M*, 99% of the enzyme activity was abolished. After being filtered through a Sephadex G-25 column,

(1) Pyridoxine Chemistry. 25. (a) Preceding paper in this series: W. Korytnyk, H. Ahrens, and N. Angelino, *Tetrahedron*, **26**, 5415 (1970); (b) a brief report of this study has appeared: 157th National Meeting of the American Chemical Society, Minneapolis, Minn., April 1969, MEDI 11.

(2) For a review of the synthesis and biological activity of vitamin B_6 analogs see W. Korytnyk and M. Ikawa, *Methods Enzymol.*, **18A**, 524 (1970).

(3) Two vitamin B_6 analogs that have bifunctional alkylating groups in the 5 position have been described earlier and found to have weak antitumor effects: N^6,N^6-bis(2-chloroethyl)-O^4-methylisopyridoxamine (methoxypyridoxyl N mustard) and N^6,N^6-bis(2-chloroethyl)-4-deoxyisopyridoxamine (4-deoxypyridoxyl N mustard) [C. C. Stock, S. Buckley, K. Suguira, and C. P. Rhoads, *Cancer Res.* **11**, 432 (1951)].

(4) (a) W. Korytnyk, E. J. Kris, and R. P. Singh, *J. Org. Chem.*, **29**, 574 (1964); (b) W. Korytnyk, *J. Med. Chem.*, **8**, 112 (1965); (c) W. Korytnyk, B. Paul, A. Bloch, and C. A. Nichol, *ibid.*, **10**, 345 (1967).

(5) Attempted preparation of the acid chloride XIV from the acid XIII according to the method of I. Tomita, H. G. Brooks, and D. E. Metzler [*J. Heterocycl. Chem.*, **3**, 178 (1966)], gave the hydrochloride of the starting material XIII. Reaction with oxalyl chloride was also negative. Compare the unsuccessful efforts at obtaining acid chlorides from pyridine carboxylic acids by E. Wenkert, F. Haglid, and S. L. Mueller, *J. Org. Chem.*, **34**, 247 (1969).

(6) B. R. Baker, D. V. Santi, J. K. Coward, H. S. Shapiro, and J. H. Jordaan, *J. Heterocycl. Chem.*, **3**, 434 (1966).

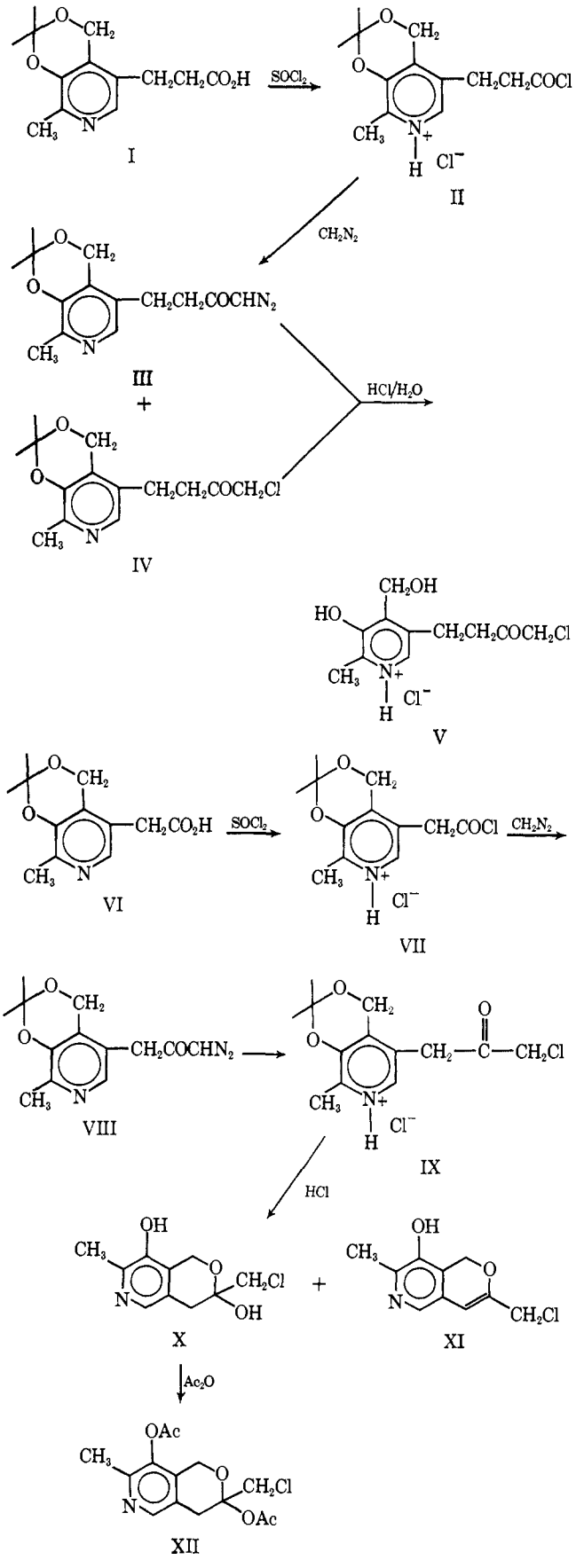
(7) (a) Crystalline apotryptophanase was prepared according to the method of W. A. Newton, Y. Morino, and E. E. Snell, *J. Biol. Chem.*, **240**, 1211 (1965); (b) compd XV was synthesized by C. Iwata and D. E. Metzler, *J. Heterocycl. Chem.*, **4**, 319 (1969).

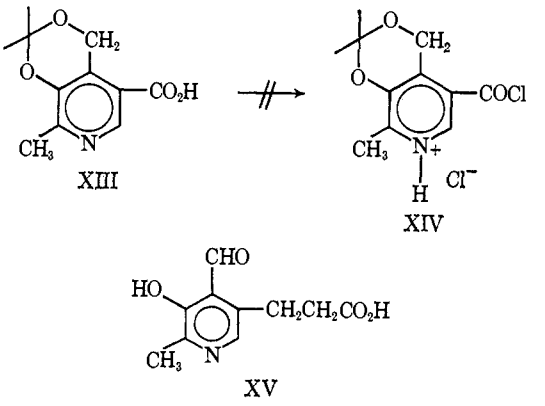
(8) The assay method was that of W. A. Newton and E. E. Snell, *Proc. Nat. Acad. Sci. U. S.*, **48**, 1431 (1962).

the enzyme had, in addition to the protein uv-absorption peak at 280 mμ, a small peak at 325 mμ for the cofactor analog, indicating covalent binding to the enzyme. In this connection it is of interest that a similar compd (but without a reactive group), β-(2-methyl-3-hydroxy-4-formylpyridin-5-yl)propionic acid (XV), was found to be a weak inhibitor of this enzyme.[7] This enzyme apparently requires an analog with a strongly ionizable group in the 5 position, such as the phosphate group, to be an effective inhibitor. Such a group is absent in V, and even the 5-carboxylic acid group in XV apparently cannot effectively simulate the phosphate group of the cofactor.

Pyridoxamine–oxaloacetic transaminase[9] was not inhibited by the analog V at 4×10^{-2} *M*, nor was pyridoxal phosphokinase[10] inhibited at 2×10^{-4} *M*. Pyridoxine phosphate oxidase from rabbit liver was also found to be uneffected at 10^{-4} *M*. The compd inhibited half-maximal growth of *Saccharomyces carlsbergensis* at 5×10^{-3} *M*; under similar conditions, the organism was inhibited by 4-deoxypyridoxol at 5×10^{-5} *M*.[11]

SCHEME I





XIII XIV XV

Experimental Section

Where analyses are indicated only by symbols of the elements, analytical results obtained for these elements were within ±0.4% of the theoretical values. Tlc (silica gel) was used routinely as described earlier.[12] Ir spectra were determined with a Perkin-Elmer 457 spectrophotometer, and nmr spectra with a Varian A60A instrument, as 8–15% solns in $CDCl_3$, DMSO, or D_2O; positions of peaks are expressed in cps from TMS, or from sodium 3-(trimethylsilyl)-1-propanesulfonic acid, as an internal std. Peaks were assigned on the basis of previous work.[13]

α^4,3-*O*-Isopropylidene-α^5-pyridoxylacetyl Chloride Hydrochloride (II).—Compd I (1.37 g, 5.5 mmoles)[4c] was suspended in C_6H_6 (20 ml, dry). $SOCl_2$ (1.2 g, 10 mmoles) in C_6H_6 (3 ml) was added dropwise to the stirred suspension, and stirring was contd for 30 min at room temp and for another 30 min at 50°. After cooling, the mixt was filtered, and the ppt was washed with dry C_6H_6. The yield was 1.5 g (91%); λ_{max}^{Nujol} 1790 cm^{-1}. The product was used for the reaction about to be described, without further purification.

1-(α^4,3-*O*-Isopropylidene-α^5-pyridoxyl)-3-diazo-2-propanone (III) **and 3-Chloro-1-(α^4,3-*O*-isopropylidene-α^5-pyridoxyl)-2-propanone** (IV).—A suspension of II (1.5 g, 4.9 mmoles) in Et_2O (10 ml) was slowly added, for approx 20 min, to a stirred and cooled (ice–salt mixt) soln of CH_2N_2 (36 mmoles, EtOH free) in Et_2O (160 ml). After standing for 12 hr, the soln was filtered and the filtrate was evapd to 15 ml and kept at 2° overnight. The deposited crystals (830 mg) consisted of III and IV, which

(9) The enzyme was prepared by J. E. Ayling and E. E. Snell, *Biochemistry*, **1**, 1616 (1968), and assayed by the method of H. Wada and E. E. Snell, *J. Biol. Chem.*, **237**, 127 (1962).

(10) From rat liver, as described by D. B. McCormick, M. E. Gregory, and E. E. Snell, *ibid.*, **236**, 2076 (1961).

(11) Dr. A. Bloch, personal communication.

(12) H. Ahrens and W. Korytnyk, *Methods Enzymol.*, **18A**, 489 (1970).

(13) W. Korytnyk and H. Ahrens, *ibid.*, **18A**, 475 (1970).

were sepd on a silica gel column (1 × 45 cm) and eluted with EtOAc; 100 mg of the mixt gave 20 mg of the α-chloro ketone IV (mp 129°), which was eluted first (indicated by positive Baker's test) and 75 mg of the α-diazo ketone III (mp 69°).

Data for diazo ketone III were: ir λ_{max}^{Nujol} 2125 (diazo), 1655 cm^{-1} (C=O); nmr ($CDCl_3$) 142 (2-CH_3), 228 (4-CH_2), 155–165 (m) (5-CH_2CH_2), 312 ($COCHN_2$), 92 (CH_3, isopropylidene), 468 (C6-H); uv λ_{max}^{EtOH} 248 mμ (ε 13,200), 277 (sh, 8600). *Anal.* ($C_{14}H_{17}N_3O_3$) C, H, N.

Data for α-chloro ketone IV were: ir: λ_{max}^{Nujol} 1740 cm^{-1}; nmr ($CDCl_3$) 142 (2-CH_3), 289 (4-CH_2), 470 (C6-H), 92 (CH_3, isopropylidene), 158–177 (m) (5-$(CH_2)_2$), 242 (5-$COCH_2Cl$). *Anal.* ($C_{14}H_{18}NClO_3$) C, H, Cl, N.

3-Chloro-1-(α5-pyridoxyl)-2-propanone Hydrochloride (V).—To the crude CH_2N_2 reaction product (III and IV, 200 mg), dissolved in Et_2O (10 ml), 1 g of concd aq HCl was added within 20 min, and the mixt was stirred at room temp. After standing for 3 hr, the solvent was evapd *in vacuo*, and the oily residue was taken up in a small amt of MeOH and shaken with Darco. After filtration and evapn of the soln, a small amt of MeCN was added till turbidity developed and let crystallize. The yield was 135 mg (60%), mp 149°. The compd gave a positive Baker's test:[6] nmr (DMSO-d_6) 157 (2-CH_3) 289 (4-CH_2), 180 (5-$(CH_2)_2$), 488 (C6-H), 273 ($COCH_2Cl$). *Anal.* ($C_{11}H_{15}Cl_2NO_3$) C, H, Cl, N.

2,2,8-Trimethyl-4*H*-3-dioxine[4,5-*c*]pyridine-5-acetyl Chloride Hydrochloride (VII).—To a stirred suspension of VI (500 mg, 2.1 mmoles) in CH_3CN (5 ml), $SOCl_2$ (600 mg, 5 mmoles) was added dropwise in *ca.* 5 min. After stirring for 15 min at room temp, the mixt was heated to 50° and was kept at this temp for 30 min. The cooled soln was filtered, and the filtrate was evapd to dryness. The residue crystd after being refluxed with dry Me_2CO. The yield was 350 mg (57%): mp 210–212° dec; ir λ_{max}^{KBr} 1805 cm^{-1} (C=O).

2,2,8-Trimethyl-4*H*-3-dioxino[4,5-*c*]pyridine-5-(3-diazo-2-propanone) (VIII).—The acid chloride VII (380 mg, 1.3 mmoles) was suspended in Et_2O (5 ml), and the suspension was added drop by drop to a stirred CH_2N_2 soln (8–10 mmoles, alcohol free) cooled to −15° with an ice–salt mixt. The soln was filtered to remove a small amt of tarry material. Tlc (EtOAc) of the filtrate showed only 1 spot. After keeping for 45 min at room temp, the reaction mixt was evapd to dryness, and the product was crystd from Et_2O–petr ether, yielding 275 mg (81%) of pale yellow crystals: mp 70°; ir λ_{max}^{KBr} 2110 cm^{-1} (N_2), 1630 cm^{-1} (C=O); nmr ($CDCl_3$) 144 (2-CH_3) 92 (CH_3, isopropylidene) 288 (4-CH_2), 208 (5-CH_2), 314 ($COCHN_2$), 474 (C6-H). *Anal.* ($C_{13}H_{15}N_3O_3$) C, H, N.

2,2,8-Trimethyl-4*H*-3-dioxino[4,5-*c*]pyridine-5-(3-chloro-2-propanone) Hydrochloride (IX).—The diazo ketone VIII was prepd, as just described, from 380 mg (1.3 mmoles) of the acid chloride VII. The filtered ethereal soln of VIII was evapd to a small vol, and the latter was slowly added to a slight excess of ethereal HCl soln (dry), with stirring. The reaction mixt was stirred for another 15 min, and was kept at 2° overnight. Filtration and washing with a small amt of dry Me_2CO yielded 240 mg (60%) of IX, mp 205° (from Me_2CO). It gave a positive Baker's test:[6] ir λ_{max}^{KBr} 1723 cm^{-1}; nmr (DMSO-d_6) 154 (2-CH_3), 93 (CH_3, isopropylidene), 285 ($COCH_2Cl$), 250 (5-CH_2CO), 298 (4-CH_2), 493 (C6-H). *Anal.* ($C_{13}H_{17}Cl_2NO_3$) C, H, N.

3-(Chloromethyl)-7-methyl-1,4-dihydropyrano[4,3-*c*]pyridine-3,8-diol (X) and the By-Product (XI).—Compd IX (61 mg, 0.2 mmole) was dissolved in 0.2 *N* HCl (7 ml), and the soln was stirred at room temp for 25 hr. Tlc indicated the formation of a new compd (R_f 0.42 in 80:20 $CHCl_3$–MeOH), giving a pos Gibbs test.

The solvent was evapd to dryness, and the residue was taken up in MeOH and again spotted on tlc. In addition to the previous spot, another spot (R_f 0.72) was obtained, which was also Gibbs pos.

The 2 products were separated by prep tlc. The compd with the lower R_f value (0.42) was extd from the tlc scrapings with MeOH. The MeOH soln was evapd, and the residue was treated with Me_2CO, giving 25 mg (54%) of product (X), mp 189 (from Me_2CO–MeOH). Baker's test on the compd was negative and the compd was not retarded by boric acid strip on tlc plate,[12] indicating that the 4-CH_2OH group is not free. Its ir spectrum shows no CO absorption; nmr (DMSO-d_6) 141 (7-CH_3), 466 (C5-H), 285 (C1-H_2), 221 (C4-H_2), 170 (3-CH_2Cl) (doublet, J = 3 cps). *Anal.* ($C_{10}H_{12}ClNO_3$) C, H, N, Cl.

The by-product of high R_f value (0.72) was isolated from the plate, but the small amt of material obtained (15 mg) was not adequate to establish the structure unequivocally as 3-(chloromethyl)-7-methyl-1*H*-pyrano[4,3-*c*]pyridin-8-ol (XI): ir λ_{max}^{KBr} 1640 cm^{-1} (C=C); nmr (DMSO-d_6) 141 (CH_3), CH_2 groups (singlets) at 257 and 312, 1 H peaks at 367 and 462 cps.

The by-product is formed directly from compd X by treatment with 0.2 *N* HCl. Tlc of the product indicated a mixt of XI and X after 1 day at room temp. It was impossible, however, to achieve complete conversion of X to XI. Likewise a mixt of X and XI was obtd when the diazo ketone VIII was treated with 38% HCl.

3-(Chloromethyl)-7-methyl-1,4-dihydropyrano[4,3-*c*]pyridine-3,8-diol Diacetate (XII).—Compd X (35 mg, 0.15 mmole) was dissolved in a 4:1 mixt of pyridine and Ac_2O, and the resulting mixt was kept at room temp for 3 days. It was evapd *in vacuo*, treated with an $NaHCO_3$ soln, and extd with Et_2O. After drying ($MgSO_4$), the EtOAc was removed *in vacuo*, and the residual oil was dissolved in Et_2O–petroleum ether. The yield of cryst material was 25 mg (53%): mp 112–113° (from Et_2O–petr ether); ir λ_{max}^{KBr} 1740, 1760 cm^{-1} (C=O); nmr ($CDCl_3$) 494 (5-H), 142 (7-CH_3), 139 (8-$OCOCH_3$), 118 (3-$OCOCH_3$), 118 (1-H_2) (s), 254 (3-CH_2Cl) (d, J = 11 cps), 239 (d, J = 11 cps), 211 (4-H_2) (d, J = 17 cps) 181 (d, J = 17 cps). *Anal.* ($C_{14}H_{16}NClO_5$) C, H, N.

Acknowledgments.—This study was supported in part by research grants (CA-08793 and CA-11047) from the National Cancer Institute, U. S. Public Health Service. We would like to thank Dr. A. Bloch under whose supervision the microbiological testing of compound V was performed. One of the authors (W. K.) is greatly indebted to Dr. Esmond E. Snell for an opportunity to carry out experiments with enzymes in his laboratory under his guidance. Tests on pyridoxine phosphate oxidase and pyridoxal phosphokinase were carried out by Mr. Norman Angelino at this laboratory.

Microsomal 3-Hydroxylation of 1,4-Benzodiazepines[1]

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Received December 8, 1970

Liver microsomal oxidations of a variety of substrates are known to be mediated by a mixed function oxygenase system which utilizes molecular oxygen and requires NADPH as a reducing equivalent.[3] In the case of tertiary amines **1** it has been proposed that microsomal oxygenation leads to the formation of a carbinolamine **2**, which, because of its inherent instability, decomposes spontaneously to the observed products, the secondary amine **3** and the aldehyde **4**.[4] Evidence consistent with this pathway was recently reported by McMahon[5] who studied the incorporation of ^{18}O-enriched O_2 into benzaldehyde formed from the microsomal oxidative dealkylation of 1-benzyl-4-phenyl-4-carbethoxypiperidine. In order to minimize exchange

(1) Presented in part at the 160th National Meeting of the American Chemical Society, Chicago, Ill., Sept 1970. Financial support from U. S. Public Health Service Grant GM 16496 is gratefully acknowledged.

(2) NATO Postdoctoral Research Fellow, 1969–1970.

(3) A recent symposium on this subject has been published: "Microsomes and Drug Oxidations," J. R. Gillette, Ed., Academic Press, New York, N. Y., 1969.

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(5) R. E. McMahon, H. W. Culp, and J. C. Occolowtz, *J. Amer. Chem. Soc.*, **91**, 3389 (1969).