Lehninger, A. L. (1960a), *Ann. N. Y. Acad. Sci. 86*, 484.
Lehninger, A. L. (1960b), *Biochim. Biophys. Acta 37*, 387.
Lehninger, A. L. (1961), *in* Proceedings of the First IUB/IUBS International Symposium, Goodwin, T. W., and Lindberg, O., eds., New York, Academic, p. 31.
Lehninger, A. L., and Schneider, M. (1958), *Z. Physiol. Chem. 313*, 138.
Leonhauser, S., Leybold, K., Krisch, K., Staudinger, Hj., Gale, P. H., Page, A. C., Jr., and Folkers, K. (1962), *Arch. Biochem. Biophys. 96*, 580.
Mackler, B., and Green, D. E. (1956), *Biochim. Biophys. Acta 21*, 1.
Michels, R., Cason, J., and Sokoloff, L. (1963), *Science 140*, 1417.
Moury, D. N. (1964), Ph.D. dissertation, Purdue University.
Moury, D. N., and Crane, F. L. (1964), *Biochem. Biophys. Res. Commun. 15*, 442.
Pederson, S., Tata, J. R., and Ernster, L. (1963), *Biochim. Biophys. Acta 69*, 407.
Pitt-Rivers, R., and Tata, J. R. (1960), The Thyroid Hormones, New York, Pergamon, p. 90.
Slater, E. C., and Cleland, K. W. (1953), *Biochem. J. 53*, 557.
Tata, J. R. (1963), *Nature 197*, 1167.
Tata, J. R., Ernster, L., Lindberg, O., Arrhenius, E., Pedersen, S., and Hedman, R. (1963), *Biochem. J. 86*, 408.
Weiss, W. P., and Sokoloff, L. (1963), *Science 140*, 1324.
Wharton, D. C., and Griffiths, D. E. (1962), *Arch. Biochem. Biophys. 96*, 103.

Activated Hydrogens in Compounds Related to Thiamine. II*

WALTER H. HAFFERL, ROBERT E. LUNDIN, AND LLOYD L. INGRAHAM*

From the Department of Biochemistry and Biophysics, University of California, Davis, and the Western Regional Research Laboratory, Albany, Calif.

Received March 9, 1964

Two model compounds were studied in order to determine whether the rapid exchange at the two position of thiamine was because of the aromaticity of the thiamine molecule or because of its cyclic structure. The unstable *N*-methyl-2-thiazolinium methyl sulfate was found to hydrolyze in tritium-enriched water with subsequent methylation to give the product *S*-methyl-*N*,*N*-dimethyl-*N*-formyl-β-aminoethanol. This product contained nonexchangeable tritium, which demonstrates that the thiazolinium salt must have very rapidly exchanged protons with solvent prior to hydrolyses. However, the hydrogens on the open-chain analog *S*-methyl-*N*,*N*-dimethylthioformimidinium iodide have an unmeasurably high half-life for exchange with protons of solvent. These results show that the rapid exchange of the proton at the two position in thiamine is dependent upon ring formation but not on aromaticity. This result can be explained by carbene character to the intermediate carbanion. The aromatic character in the thiazolium ring of thiamine is not necessarily for carbanion formation but is necessary for other further reactions of the thiamine.

Carbanion formation in the thiazolium ring of thiamine is the first step in all mechanisms (Breslow, 1958; White and Ingraham, 1962) that may be written for reactions catalyzed by thiamine in biological systems. A carbanion is surprisingly easy to form in thiazolium salts. Breslow found that the half-life of the hydrogen at the 2- position of 3,4-dimethylthiazolium iodide was on the order of minutes in neutral D_2O. This paper gives a second report of studies designed to learn the reason for this unexpected high acidity in thiazolium ions. The previous report (Hafferl *et al.*, 1963) on the H-exchange rate in thiazolium, oxazolium, and imidazolium salts, and on the lack of exchange in noncyclic quaternized Schiff bases showed that neither sulfur-shell expansion nor sp^2 hybridization of a carbon adjacent to a charged nitrogen causes this high rate of H exchange.

The present study was undertaken to determine whether the exchange might be a result of the aromaticity or cyclic structure of all models which were found to exchange earlier. Experiments with a partially hydrogenated thiazole and its open-chain analog were performed to study the influence of both aromaticity and ring structure.

* We wish to acknowledge support of this project by a grant (GM 8285) from the United States Public Health Service.

EXPERIMENTAL

Preparations

2-Thiazoline (I).—This compound was synthesized by the method of Wenker (1935). Some decomposition of the crude base was caused by the potassium hydroxide drying agent. The purified compound was stable to metallic sodium so that the last traces of water were removed by high-vacuum distillation over sodium. It boiled at 38–42° (13 mm Hg) and at 139° (760 mm Hg). The infrared absorption is listed in Table I.

N-Methyl-2-thiazolinium Methyl Sulfate.—Three g of thiazoline (freshly distilled in high vacuum over sodium) was diluted with 15 ml benzene (dried over sodium). The solution was kept at 0° as 4.4 g of dimethyl sulfate (twice-distilled) was added. Soon a crystalline precipitate formed, which was allowed to stand 0.5 hour at 0°. The reaction product was very unstable in all polar solvents and even in benzene at room temperature. It was used immediately without further purification or identification.

S-Methyl-N,N'-dimethyl-N-formyl-(^3H)-β-aminoethanol (III).—The methylated thiazoline (II) was added to a solution of 12.5 g of dibasic potassium phosphate and 10 g monobasic potassium phosphate in 20 ml of tritiated water (1 mc/ml). The solution was shaken and almost immediately droplets of an oil appeared. The *p*H dropped from 9.4 to 7.3 during the few seconds of shak-

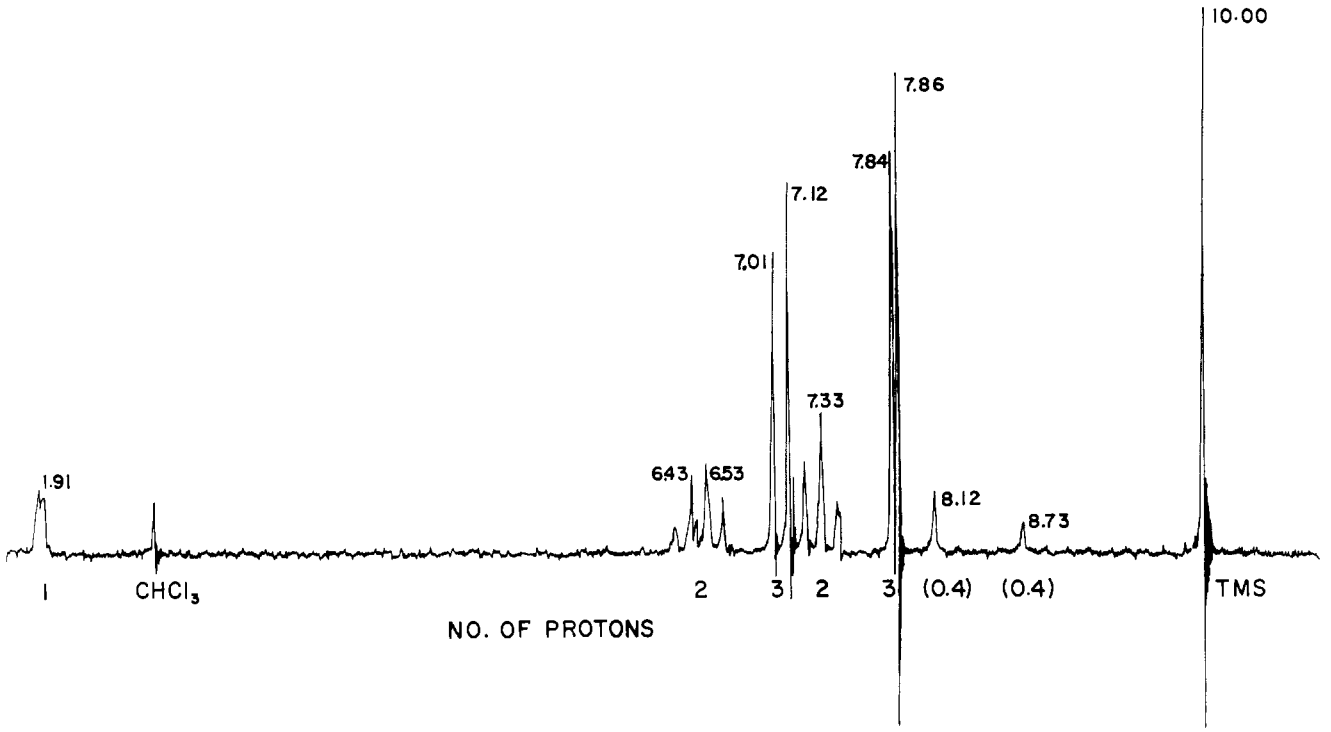
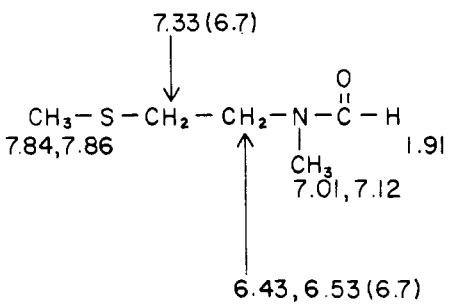
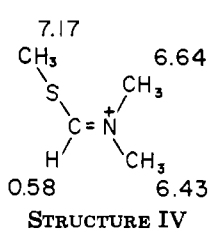


FIG. 1.—The NMR spectrum at 60 Mc of a 5% solution of *S*-methyl-*N*-methyl-*N*-formyl-β-aminoethanol in *d*-chloroform. The shielding values in τ units refer to tetramethyl silane, τ = 10, and the spin-spin coupling constants (in parenthesis) in cps. The double peaks are the result of two conformers corresponding to those of *N*,*N*-dimethylformamide.



STRUCTURE III



STRUCTURE IV

TABLE I
INFRARED ABSORPTION OF 2-THIAZOLINE AND 2-OXAZOLINE[a]

2-Thiazoline		2-Oxazoline	
3550	very w	3500	very w
3200	w	3310	w
3100	w	3110	m
3000	st	3000	st
2900	st	2920	st
2300	very w	2190	w
1750	very w	1860	w
1610–1570	very st	1650–1610	very st
1445	st	1480	st
1305	m	1455	w
1240	very st	1342	st
1190	m	1298	st
1140	m	1110–1060	very st
1005	m	990	w
965	very st	970	m
940	w	945–910	very st
878	m	705	broad m
840–780	very st		
710	m		

[a] Pure liquid, 0.025 mm path, frequency values in cm.$^{-1}$. Key: st, strong; m, medium; w, weak.

ing. Ethanol was added to obtain a homogeneous phase. The solvent was removed *in vacuo*, and the residue was twice dissolved in 25 ml aqueous ethanol and lyophilized. The oil was extracted from the inorganic salts with 15 ml of butanol. Removal of the butanol *in vacuo* and distillation of the residue gave 1.6 g of a neutral viscous oil boiling at 95° (0.12 mm Hg). The oil was soluble in alcohol and chloroform but insoluble in water, benzene, and ether. The NMR[1] spectrum is in agreement with structure (III), contaminated with fission products of unreacted thiazoline.

Five 1-μl portions of the oil were separated on a 304.8 × 0.64-cm (10 ft × 1/4 in.) gas-liquid chromatography column with a low-polar stationary phase (Apiezon or Carbowax on firebricks) at 150°. Helium (100 ml/m) was used as carrier gas. The eluate of the main peak was fractionally collected in a glass tube (*ca* 2 mm wide, 10 cm long), which was cooled with wet paper. If the injected amount did not exceed 5 μl, the loss by fogging was small. After five collections one end of the tube was closed by melting, and the substance that coated the inner surface of the tube was centrifuged to the bottom of the tube.

Anal. Calcd for $C_5H_{11}NOS$: C, 45.08; H, 8.32. Found: C, 45.51; H, 7.89. (The NMR spectrum indicates minor hydrocarbon impurities from the column.) To obtain material for spectroscopy the eluate was collected in very good yield on glass wool,

[1] Abbreviation used in this work: NMR, nuclear magnetic resonance.

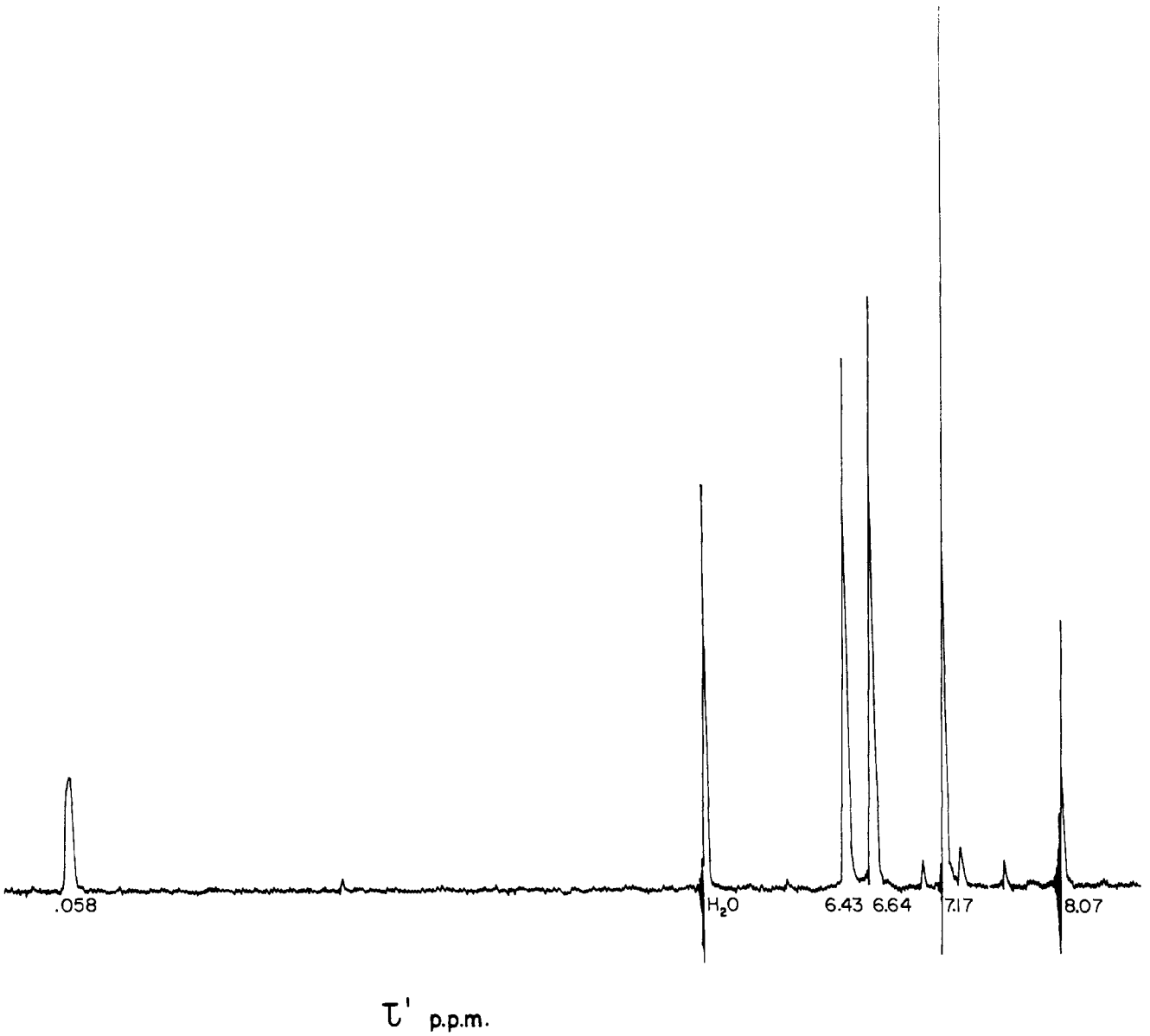


FIG. 2.—The NMR spectrum at 60 Mc of a 5% solution of *S*-methyl-*N*,*N*-dimethylthioformimidinium iodide (IV) in 0.1 N sodium acetate buffer at *p*D 5.6 in D_2O. The shielding values, τ', are with internal 3-(trimethylsilyl)-1-propanesulfonic acid taken as 10.00.

and moistened with *d*-chloroform. The eluate was extracted from the glass wool by centrifugation and washing with *d*-chloroform. The NMR spectrum (Fig. 1) of the pure material agrees with structure (III). A time of flight mass spectrum showed a parent peak at mass 133 (±0.3) in agreement with the calculated molecular weight 133.22.

Liquid Scintillation Counting

After vacuum distillation, 26.3 mg of product III was dissolved in 10 ml of a dioxane-naphthaline scintillation solution (Bray, 1960) and radioactivity was counted on a Packard Tri-Carb liquid scintillation spectrometer.[2] One mg gave 2020 cpm. After further purification by gas-liquid chromatography on an Apiezon column, 2.47 mg gave 4336 cpm (average of 5 values), or 1718 cpm per mg. Addition of standard [^{3}H]toluene showed a counting efficiency of 11%. The molar activity of (III) is 0.96 mc, i.e., 10% of the expected value for one totally exchanged hydrogen.

S-Methyl-N,N'-dimethylthioformimidinium Iodide (IV).—This compound was prepared by the procedure of Willstatter and Wirth (1909). The authors called the compound dimethylthioformamide iodine methylate and did not give a structure. The melting point (119–123°) is identical to that of the Willstatter and Wirth preparation. The NMR spectrum (Fig. 2) is in agreement with the structure (IV).

2-Oxazoline.—This compound was prepared by the procedure of Wenker (1938). The last traces of water and other impurities were removed by vacuum distillation over sodium wire. The boiling point was 98.6–99° at 761 mm Hg. The infrared spectrum is shown in Table I.

Nuclear Magnetic Resonance Studies of the Reaction of Compound IV with D_2O

About 20 mg of IV was dissolved in 0.6 ml 0.1 N potassium phosphate buffer *p*D 7 in D_2O, and the spectral changes were observed as a function of time. The hydrolysis caused the appearance of a formyl-proton peak (τ' = −0.11) at the same rate as the azomethine hydrogen peak decreased. The integral of both peaks remained constant during the whole experiment

[2] Mention of commercial products does not constitute endorsement by the U. S. Department of Agriculture.

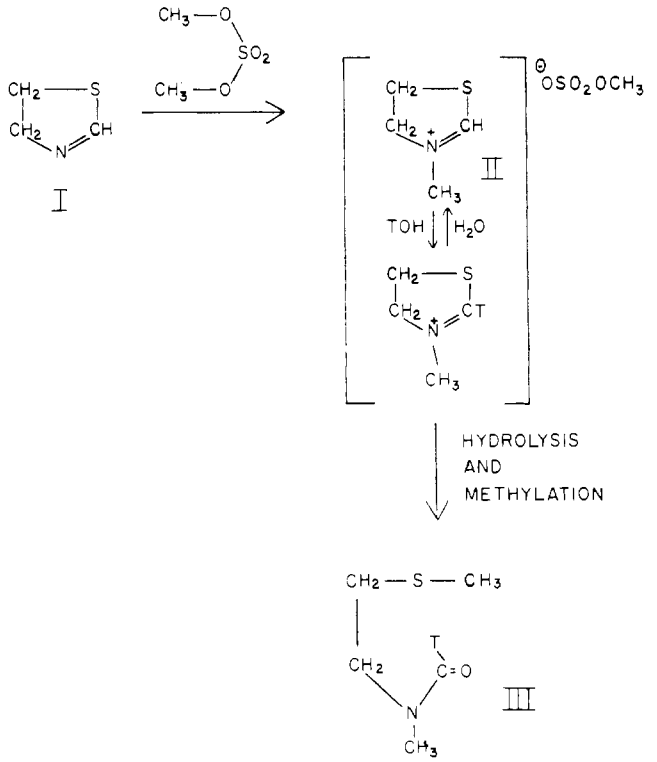


FIG. 3.—Reaction sequence of 2-thiazoline (I). Methylation of 1 with dimethyl sulfate gave (II), which was methylated during hydrolysis to give (III), with about 10% of one possible tritium atom per molecule.

(58 minutes), indicating that there was no detectable H exchange. The *p*D of the solution shifted during the experiment to 3.9.

RESULTS

The very unstable *N*-methyl-2-thiazolinium methyl sulfate was found to hydrolyze to a labeled compound in tritiated water as shown in Figure 3. The product isolated was the *S*-methyl ether of the expected product. The first hydrolysis product must have been methylated subsequently by the CH_3–OSO_3^- ion. The fact that this compound contains a nonexchangeable tritium shows that the thiazolinium salt had an activated proton. By analogy with thiazolium ions it is likely to be in the 2-position. The hydrolyzed product had incorporated about 10% of the possible amount of one proton. This indicates that the rate of exchange is 10% of the very fast rate of hydrolysis. The instability of the thiazolinium compound did not permit a determination of the rate of hydrolysis. Attempts to perform the same experiments with oxazolinium salts failed because a crystallized methylation product was not obtained.

The methylation product of *N*,*N*-dimethylthioformamide was assigned the structure *S*-methyl-*N*,*N*-dimethylthioformimidinium iodide (IV) because of the *S*-methyl peak in the NMR spectrum (see Fig. 2). This compound is the acyclic analog of thiazolinium iodide, but unlike thiazolium and thiazolinium ions it showed no evidence of hydrogen exchange in D_2O at an average *p*D of 5.5. The error limit (±5%) indicates that if any exchange occurs the half-life of this exchange must be longer than 15 hours.

DISCUSSION

The observation that thiazolinium salts will exchange protons with solvent shows that the full aromaticity of the thiazolium ring is not required for proton acidity. Surprisingly, the open-chain analog of the thiazolinium ring showed no proton exchange with solvent. The hydrolysis rate was also much less in the open-chain analog. This means that the double bond must be under some strain in the thiazolinium ion and suggests that this strain also contributes to the exchange rate. The stability of the double bond would contribute to the exchange rate if the carbanion had some carbene character (Fig. 4), as suggested by Breslow (1958) to explain the high rate of exchange in thiazolium salts and later by Wanzlick and Schikora (Wanzlick, 1962; Wanzlick and Schikora, 1961) to explain the purported dissociation of bis-(1,3-diphenylimidazolidinylidene-2).[3]

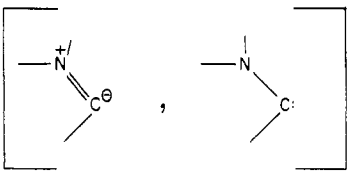


FIG. 4.—Carbanion (Ylenid)–carbene resonance.

All relatively stable carbanion intermediates possessing carbene character that have been found to date (including the thiamine carbanion) have been cyclic (Breslow, 1958; Wanzlick and Schikora, 1961; Wanzlick, 1962; Hafferl *et al.*, 1963). This may be a general rule because any carbanion-carbene resonance would require that the carbene be a singlet. A singlet carbene probably has sp^2 hybridization and therefore requires a bond angle of 120°. Possibly, the lone electron pair on the carbene may not be sufficient to hold this structure. An analogy would be amines in which the lone electron pair on nitrogen is not sufficient to hold the nitrogen in a tetrahedral configuration.

Although the cyclic structure of the thiamine may be important for carbanion formation, the aromaticity is unnecessary. The aromaticity of thiamine is important (Hafferl *et al.*, 1963) for both carbanion formation on the substrate and for stability to hydrolysis.

A comparison of the rate of exchange of the proton at the 2- position of oxazolium and thiazolium salts with the corresponding ^{13}C-H NMR-coupling constants led Haake and Miller (1963) to propose that *d*-σ overlap also contributes to the stability of thiazolium ylenids. Our data gives no information as to the importance of this factor but by no means does it eliminate the importance of *d*-σ overlap.

ACKNOWLEDGMENT

We want to express our thanks to Dr. W. G. Jennings and co-workers for assistance in the gas-liquid chromatography experiments, and to Dr. William McFadden for performing the mass spectrometry.

REFERENCES

Bray, G. A. (1960), *Anal. Biochem. 1*, 279.
Breslow, R. (1958), *J. Am. Chem. Soc. 80*, 3719.

[3] Attempts to synthesize the corresponding dimerization product of a thiazolium salt were unsuccessful. Proton abstraction from *N*-methylthiazolium iodide with mesitylmagnesium bromide in pyridine at 80° under nitrogen and decomposition with hydrochloric acid did not yield the expected bis-(*N*-methyl-4-thiazolinylidene-2), but *N*-methyl-4-thiazoline-thione-2, mp 47°. This structure is in agreement with the NMR spectrum and the analyses. Calcd for $C_4H_5NS_2$: C, 36.61; H, 3.84; N, 10.68; S, 48.87. Found: C, 36.84; H, 4.07; N, 10.45; S, 48.52.

Haake, P., and Miller, W. B. (1963), *J. Am. Chem. Soc. 85*, 4044.
Hafferl, W., Lundin, R. E., and Ingraham, L. L. (1963), *Biochemistry 2*, 1298.
Wanzlick, H. W. (1962), *Angew. Chem. 74*, 129.
Wanzlick, H. W., and Schikora, E. (1961), *Ber., 94*, 2389.
Wenker, H. (1935), *J. Am. Chem. Soc. 57*, 1079.
Wenker, H. (1938), *J. Am. Chem. Soc. 60*, 2152.
White, F. G., and Ingraham, L. L. (1962), *J. Am. Chem. Soc. 84*, 3109.
Willstatter, R., and Wirth, T. (1909), *Ber. 42*, 1920.

Synthesis and Immunochemistry of Fucose Methyl Ethers and Their Methylglycosides*

GEORG F. SPRINGER, PARIMAL R. DESAI, AND BEN KOLECKI

From the Department of Immunochemistry Research,† Evanston Hospital Association, and the Department of Microbiology, Northwestern University, Evanston, Ill.

Received March 18, 1964

L-Fucopyranose in α-glycosidic linkage has been thought to be responsible for blood-group-H(O) specificity of human blood-group mucoids as determined with heterologous anti-H(O) reagents from the eel and *Lotus tetragonolobus*. This conclusion was based on hapten studies. However, some L- and D-fucose-*O*-methyl ethers were as active as L-fucose. Therefore the stereospecific requirements for the activities of fucose methyl ethers and their methylglycosides were systematically investigated. Eight fucose-*O*-methyl ethers and fourteen methylglycopyranosides of fucose were synthesized and characterized. The majority of these sugars were crystallized and ten are novel. Quantitative precipitin-inhibition tests in the eel serum anti-H(O)—H(O) substance system were only in qualitative accord with hemagglutination-inhibition tests. A number of methylated L- and D-fucoses and their methylglycopyranosides were potent inhibitors. The enantiomorphs of 3-*O*- and 2,3-di-*O*-methylfucoses had nearly identical activities. 3-*O*-Methylfucose precipitated the anti-H(O) antibody of some eel sera and may thus be the smallest uncharged antibody-precipitating hapten yet found. The complementary structure for the eel serum antibody is probably smaller than a monosaccharide. It seems to consist of a methyl substituent attached equatorially to a pyranose; there is an ether oxygen adjoining the methyl and an axial, oxygen-carrying substituent *cis* to the methyl group is on a contiguous C atom. An *O*-methyl at C-4 is compatible with activity. Hapten requirements for activity in the *Lotus* system differ; a substituent at C-2 is a precondition in the D series. A furanoid structure substituted with three *O*-methyl groups is equally active in both series. While appropriate methyl substitution increases the activity of highly active fucose compounds, it can inactivate slightly active fucose ethers.

Simple substances which combine specifically with antibodies or antibodylike reagents are thought to be identical with or very closely related to the immunologically determinant group of an antigen. This concept is based on Landsteiner's classical studies (1920). More recently it has been successfully applied to the elucidation of the nature of the antigenically determinant groupings of blood-group-specific substances (cf. Kabat, 1956; cf. Morgan, 1960).

It was first observed by Morgan and Watkins (1953) that L-fucose inhibited the agglutination of human blood-group-O erythrocytes by anti-H(O) agglutinins in eel serum and in seed extracts of *Lotus tetragonolobus*. This inhibition was thought to possess considerable specificity in that L-fucose was the only active sugar of the four constituent monosaccharides of blood-group mucoids. Kuhn and Osman (1956) confirmed and extended the earlier findings of Morgan and Watkins and postulated the following necessary conditions for the serological activity of fucosides: (a) The fucose residue must belong to the L series; (b) it must be in the pyranose form; (c) if linked, it must be present in α-glycosidic linkage. Other sugars were thought to be inactive.

Springer *et al.* (1956) reported that the polysaccharide with high blood-group-H(O) activity isolated from *Taxus cuspidata* contained no L-fucose, but the serologically active sugar was 2-*O*-methyl-L-fucose, not then known to occur in nature.

Earlier studies (Springer and Williamson, 1962) had shown that previously held ideas on the stereospecificity of serological reactions do not apply to the eel serum antibody and apply only to a limited extent to the *Lotus tetragonolobus* agglutinin, at least if the smallest structure which is complementary to the combining site of an antibody is considered to be a monosaccharide. While L-fucose and some of its derivatives are highly active substances, high and sometimes equal activities are also found among substituted members of the D series in both hemagglutination and precipitation-inhibition tests. Kabat (1962) has offered an explanation for these observations of equal biological activity of enantiomorphs.

These studies suggested limitations of heterologous serological reagents for the prediction of the nature of immunochemically specific groups. The ultimate rigid chemical characterization of serologically specific structures, therefore, is indispensable, especially in the assessment of blood-group substances, since experimental evidence has been adduced (Springer *et al.*, 1959, 1962; for theoretical aspects of this problem see

* This investigation has been supported by a grant (GB-462) from the National Science Foundation. Part of this work has been presented by the authors at the National Meetings of the American Chemical Society in Spring, 1962, and Fall, 1963.

† Supported by Susan Rebecca Stone Fund for Immunochemistry.