

Figure 1. Pressure dependence of the visible spectrum ( $20^\circ$ ) of aqueous metmyoglobin fluoride ( $ca. 3 \times 10^{-5} M$ ; prepared from Sigma type II sperm whale myoglobin without further purification). All curves were taken after the system had reached equilibrium except for that at  $5250 \text{ kg/cm}^2$ , which was taken after 2 min.

$m\mu$  similar to that of a hemoprotein with the  $\text{Fe}^{\text{III}}$  atom of the porphyrin nucleus in a low-spin configuration.<sup>1</sup> It is characteristic of heme in which the  $\text{Fe}^{\text{III}}$  atom is coordinated to six nitrogen-containing ligands (hemochromogen),<sup>2</sup> and is virtually identical with the spectrum of metmyoglobin imidazole obtained by adding an excess of imidazole to metmyoglobin at atmospheric pressure. The spectrum of the metmyoglobin imidazole complex is essentially unaffected by pressurization to  $6500 \text{ kg/cm}^2$ . This observation suggests that the spectral change is due to a conformational change in the protein, in which the fluoride ligand is replaced by a nitrogen of an imidazole side chain in the high-pressure conformation. The possibility of a pressure-induced process such as this was suggested by Fabry and Hunt<sup>3</sup> in connection with the pressurization of hemoglobin and its derivatives to  $2000 \text{ kg/cm}^2$ . Fabry studied only shifts in the position of the Soret band. Therefore, he could not determine whether the high-spin  $\rightleftharpoons$  low-spin transition was caused by the replacement of the ligand in the sixth coordination position by imidazole (possibly that at E7) or simply by a compression of the iron–ligand bond.

We have also studied the effects of pressure on the visible absorption spectrum of methemoglobin and several of its derivatives and observe spectral changes similar to those obtained for metmyoglobin fluoride. The changes occur at considerably lower pressure ( $500$ – $1500 \text{ kg/cm}^2$  for methemoglobin fluoride) and are only partially reversible.

The results shown in Figure 1 were obtained using  $0.05 M$  cacodylate buffer at pH 6.25. It is well established that the pH of a buffer solution will change upon pressurization. From dilatometric measurements made by Dr. Frank Gasparro in this laboratory and from compressibilities estimated by the method of Lown, *et al.*,<sup>4</sup> we estimate that the pH of this cacodylate buffer

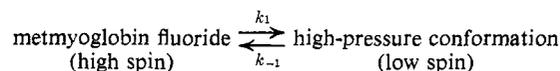
(2) D. L. Drabkin and J. H. Austin, *J. Biol. Chem.*, **112**, 89 (1935–1936).

(3) T. L. Fabry and J. W. Hunt, Jr., *Arch. Biochem. Biophys.*, **123**, 428 (1968).

(4) D. A. Lown, H. R. Thirsk, and Lord Wynne-Jones, *Trans. Faraday Soc.*, **64**, 2073 (1968).

will be lowered by no more than 1.3 units at  $6500 \text{ kg/cm}^2$ . Although this pH change will cause slight shifts in the absorption maxima, a pH 5 buffer at 1 atm does not lead to the spectral changes described above.

Since metmyoglobin fluoride was stable toward aggregation or precipitation for periods of at least 4–5 hr under the conditions employed, it was possible to obtain both rate and equilibrium constants for the process



From the effect of pressure on the equilibrium constant the overall  $\Delta V^\ddagger$  of this reaction was calculated to be  $ca. -90 \text{ cm}^3/\text{mol}$ . Apparent first-order rate constants for the forward reaction ( $k_1$ ) ranged from  $ca. 4 \times 10^{-5}$  to  $ca. 3 \times 10^{-4} \text{ sec}^{-1}$  over the pressure range  $3850$ – $4400 \text{ kg/cm}^2$ , and gave a value of  $\Delta V^\ddagger$  essentially the same as that for  $\Delta V^\ddagger$ .<sup>5</sup>

Similar studies on ribonuclease<sup>6</sup> and chymotrypsinogen<sup>7</sup> have been reported. These results were interpreted in terms of pressure-induced conformational changes, and it would appear that similar changes are taking place here. We are examining other heme proteins and extending our measurements over wider temperature, pH, and spectral ranges.

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(5) At a given pressure, spectra used to obtain rate data showed isosbestic points at 485, 507, and 580  $m\mu$  supporting the above two-state interpretation of this equilibrium. NOTE ADDED IN PROOF. Although these data have been interpreted in terms of a two-state equilibrium, subsequent kinetic studies of the reversal reaction yield biphasic plots indicating that more than two states must be involved in the kinetics.

(6) J. F. Brandts, R. J. Oliveira, and C. Westort, *Biochemistry*, **9**, 1038 (1970).

(7) S. A. Hawley, *ibid.*, **10**, 2436 (1971).

(8) Department of Chemistry, University of Ibadan, Ibadan, Nigeria.

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### Computer-Aided Mass Spectrometric Identification of Stereoisomeric Monosaccharides<sup>1</sup>

Sir:

The identification of microgram amounts of monosaccharides is an intricate problem in the structure determination of oligo- and polysaccharides because of the occurrence of a large number of stereoisomers. Although mass spectrometry has been widely applied in the field of carbohydrate research,<sup>2–4</sup> the influence of the configuration on the fragmentation is still obscure. Some authors reported a correlation between the mass spectra and the configuration,<sup>5,6</sup> whereas others indi-

(1) This work was presented in part by J. J. de R. at the Mass Spectroscopy Group Meeting, Bristol University, Bristol, Great Britain, July 13–15, 1971.

(2) N. K. Kochetkov and O. S. Chizhov, *Advan. Carbohydr. Chem.*, **21**, 39 (1966).

(3) K. Heyns, H. F. Grützmacher, H. Scharmann, and D. Müller, *Fortsch. Chem. Forsch.*, **5**, 448 (1966).

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cated that mass spectrometry is insensitive to stereochemical differences in the monosaccharides.<sup>7,8</sup>

In order to investigate the applicability of mass spectrometry for monosaccharide identification, we examined the spectra of a series of aldohexoses more closely. For this study we used the trimethylsilyl (TMS) derivatives of the aldohexopyranoses  $\alpha$ -D-glucose,  $\alpha$ -D-galactose,  $\alpha$ -D-talose,  $\beta$ -D-glucose,  $\beta$ -D-mannose,  $\beta$ -D-allose, and  $\alpha$ -L-mannose.<sup>9</sup>

The mass spectra of these stereoisomeric compounds turned out to be very similar, as might be expected on account of the uniform carbon skeleton (for a typical example of an aldohexose mass spectrum see DeJongh, *et al.*<sup>7</sup>). We have already reported for stereoisomeric disaccharides<sup>10</sup> that a close investigation of peak intensities shows minor but definite and reproducible differences. These differences become more pronounced when intensity ratios are compared. We found that the same feature holds for monosaccharides as well.

For identification of monosaccharides, we selected a set of eight intensity ratios:  $I(435)/I(393)$ ,  $I(393)/I(361)$ ,  $I(361)/I(305)$ ,  $I(345)/I(265)$ ,  $I(332)/I(319)$ ,  $I(305)/I(265)$ ,  $I(291)/I(265)$ , and  $I(265)/I(189)$ . These ratios have been selected by judgment of two criteria, *viz.* (i) the mass difference between the fragment ions may not exceed a certain value which is specified on the basis of the scan speed used; this criterion is applied in order to eliminate excessive intensity variations during the scan; (ii) the number of times the interval  $R \pm sd$ <sup>11</sup> of each intensity ratio for one compound overlaps with intervals of the corresponding ratios of any other component should be as low as possible.

Peak intensity ratios as well as their standard deviations have been determined by recording several times the individual low-resolution mass spectra<sup>12</sup> of the compounds specified above. In order to approach independent measurements, the number of sample preparations amounted to two–three per compound and each sample was introduced at least three times into the mass spectrometer. The number of scans per sample introduction (10–30  $\mu$ g) was confined to four–six, resulting in about 20–30 individual spectra per monosaccharide.

Aldohexoses are identified by matching the intensity ratios of the unknown with the corresponding data of a set of reference compounds. This matching procedure is performed by a computer program based upon the theory of multidiscriminant analyses.<sup>13</sup> Comparing the

(5) K. Heyns and H. Scharmann, *Justus Liebig's Ann. Chem.*, **667**, 183 (1963).

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(9) The authors thank Mr. G. J. Gerwig for preparation of the trimethylsilyl derivatives.

(10) V. Vink, J. J. de Ridder, J. P. Kamerling, and J. F. G. Vliegert, *Biochem. Biophys. Res. Commun.*, **42**, 1050 (1971).

(11)  $R$  refers to the arithmetic mean of the measured intensity ratios and  $sd$  stands for standard deviation. The standard deviation is computed according to the formula in: W. J. Dixon and F. J. Massey, "Introduction to Statistical Analysis," McGraw-Hill-Kogakusha, New York-Tokyo, 1957, p 19.

(12) The mass spectra have been recorded with an AEI MS 902 mass spectrometer under the following conditions: electron accelerating voltage, 70 V; ionizing current, 500  $\mu$ A; ion source temperature, 100–130°.

(13) For the theory of the multidiscriminant analyses see: C. R. Rao, "Linear Statistical Inference and Its Applications," Wiley, New

York, N. Y., 1968, pp 413–417, 436–449, 487–490; and T. W. Anderson, "An Introduction to Multivariate Statistical Analysis," Wiley, New York, N. Y., 1958, Chapter 6. The authors are indebted to Drs. F. P. G. M. La Fors for the introduction of the multidiscriminant analyses and to Drs. P. C. Sander and Miss Joke Lijnse for computer programming.

$$S_i = \left[ \sum_{n=1}^m a_n R_n \right] + C_i$$

where the summation is taken over the product of a weight factor  $a_n$  and an intensity ratio  $R_n$  of the unknown to be identified. The constant  $C_i$  is calculated from the standard data of the reference compounds. The weight factors  $a_n$  are estimated by the program on the basis of the 20–30 standard spectra, whereas the intensity ratios of the unknown can be determined by one single scan (*vide infra*). According to statistics, the compound for which the equation yields the highest sum value,  $S_i$ , is most likely to correspond with the structure of the unknown. Using this program we processed 187 individual scans (covering all seven investigated stereoisomers) by comparing them with the complete set of standard data. In 186 cases the highest  $S_i$  value corresponds with the correct identification indeed, whereas once  $\alpha$ -L-mannose was identified as  $\alpha$ -D-glucose.

As an illustration of the results, we processed the spectra of four known aldohexoses as if they were unknown. The spectra of these compounds,  $\alpha$ -D-talose,  $\beta$ -D-mannose, and  $\alpha$ -D- and  $\alpha$ -L-glucose, were not comprised in the file of reference spectra, which was recorded under identical instrumental conditions half a year before. Per compound at least four spectra have been recorded. All of them were identified correctly so that recording of one single scan probably will suffice. An example of the computer output of these analyses is shown in Table I. Processing the spectra

Table I

Saccharide	Sum	Saccharide	Sum
$\beta$ -D-Mannose		$\alpha$ -D-Talose	
$\beta$ -D-Man	133.745	$\alpha$ -D-Tal	109.461
$\alpha$ -D-Glu	119.921	$\beta$ -D-Man	85.455
$\alpha$ -D-Tal	119.305	$\alpha$ -D-Glu	78.833
$\alpha$ -D-Gal	92.784	$\alpha$ -D-Gal	71.282
$\alpha$ -L-Man	85.912	$\beta$ -D-Glu	35.518
$\beta$ -D-Glu	74.876	$\beta$ -D-Al	20.024
$\beta$ -D-Al	61.246	$\alpha$ -L-Man	14.613
$\alpha$ -D-Glucose		$\alpha$ -L-Glucose	
$\alpha$ -D-Glu	209.289	$\alpha$ -D-Glu	203.326
$\beta$ -D-Man	185.105	$\beta$ -D-Man	175.422
$\alpha$ -D-Tal	169.233	$\alpha$ -D-Tal	160.848
$\beta$ -D-Glu	161.603	$\beta$ -D-Glu	154.747
$\alpha$ -L-Man	155.008	$\alpha$ -L-Man	143.445
$\alpha$ -D-Gal	151.512	$\alpha$ -D-Gal	139.417
$\beta$ -D-Al	114.779	$\beta$ -D-Al	102.008

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to distinguish antipodes by mass spectrometry. There are indications that it is not necessary that the spectra of the unknown and those of the reference compounds are recorded using exactly the same mass spectrometer. Correct identifications have been obtained for spectra recorded by other mass spectrometers of the same design as the one used for the reference compounds.<sup>14</sup>

The potential of this new method for unequivocal identification of microgram amounts of stereoisomeric monosaccharides, which form part of the set of reference compounds, is demonstrated in these experiments. The method turned out to be reasonably fast: the total time for analysis is mainly determined by the necessary purification of the TMS derivatives and by memory effects in the mass spectrometer.

Although mixtures of TMS derivatives of monosaccharides can be fairly well separated by gas-liquid chromatography (glc), a straightforward glc identification of monosaccharides may sometimes meet with difficulties, particularly when the mixtures are composed of a large number of different monosaccharides with extreme variations in concentration. Experiments are in progress to test the value of an on-line operating glc-mass spectral computer system for analyses of monosaccharide mixtures.

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(14) We processed, for instance, the literature<sup>7</sup> spectrum of TMS- $\alpha$ -D-glucose (recorded with an AEI MS 902) as well as spectra recorded with our AEI MS 9; in both cases correct identifications have been obtained. The applicability of the identification method under different instrumental conditions and using other types of mass spectrometers will be further investigated.

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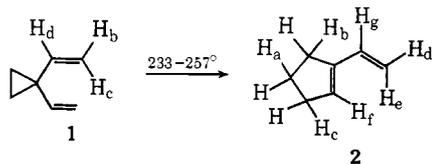
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## Thermal Reorganization of 1,1-Divinylcyclopropane

Sir:

1,1-Divinylcyclopropane was synthesized from cyclopropane-1,1-diacetic acid<sup>1</sup> via sequential (1) reduction by  $\text{LiAlH}_4$ , (2) tosylation, and (3) elimination using potassium *tert*-butoxide in DMSO at 26°. The overall yield for the process was ~45%. The structure of 1



was verified by its nmr, ir, and mass spectra and analysis. The nmr ( $\text{CCl}_4$ ) showed a sharp singlet at  $\delta$  0.79 ppm (4 H) as well as doublets at 4.89 ( $J_{bd} = 9.5$ ,  $J_{bc} = 1.7$  Hz, 2 H), 4.91 ( $J_{cd} = 18$ ,  $J_{bc} = 1.7$  Hz, 2 H), and 5.78 ppm ( $J_{bd} = 9.5$ ,  $J_{cd} = 18$  Hz, 2 H); the ir

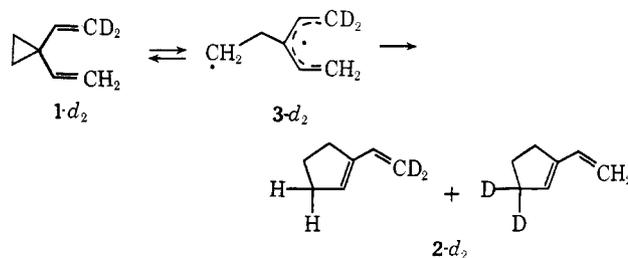
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spectrum showed strong peaks at 1670, 1000, and 808  $\text{cm}^{-1}$  and the mass spectrum at  $m/e$  94 (P), 79 (base), 77, and 39.

1 could be converted quantitatively at temperatures above 230° to 1-vinylcyclopentene:<sup>2,3</sup> nmr, a broad doublet at  $\delta$  1.93 (2 H), a broad multiplet at 2.30–2.50 (4 H), doublets at 4.94 ( $J_{dg} = 9.5$ ,  $J_{de} < 2$  Hz, 1 H) and 4.96 ( $J_{eg} = 18$ ,  $J_{de} < 2$  Hz, 1 H), a broad singlet at 5.62 (1 H), and a doublet of doublets at 6.48 ppm ( $J_{dg} = 9.5$ ,  $J_{eg} = 18$  Hz, 1 H); ir, strong peaks at 2980, 990, and 902  $\text{cm}^{-1}$ ; uv,  $\lambda_{\text{max}}$  233  $\text{m}\mu$  ( $\epsilon$  22,400).

The activation parameters for this reaction were determined in benzene solution using a temperature range of from 233 to 257°:<sup>4</sup>  $E_a = 39.6 \pm 1$  kcal/mol;  $\log A = 12.57$ ;  $\Delta H^\ddagger = 38.6 \pm 1$  kcal/mol and  $\Delta S^\ddagger = -2.8 \pm 4$  cal/deg. These results indicate that the addition of a *second* vinyl groups lowers the activation energy for the vinylcyclopropane  $\rightarrow$  cyclopentene rearrangement by ~11–12 kcal/mol. The fact that one observes an activation energy lowering almost equal to that due to the presence of the first vinyl group (~14 kcal/mol)<sup>5,6</sup> could be construed as being consistent with either a concerted process or the intermediacy of a diradical species in the reaction.

Our earlier work on the (2 + 2) cycloadditions of 1,1-dideuterioallene<sup>9</sup> indicated that the product-forming cyclization process of an intermediate diradical can give rise to a significant normal secondary deuterium isotope effect. In a similar manner, the intermediacy of a diradical 3 in the conversion of 1  $\rightarrow$  2 was probed utilizing the dideuterated and tetradeuterated species 1- $d_2$  and 1- $d_4$ . Using quantitative



integrations of 2- $d_2$ , the value of the *intramolecular* isotope effect for the reaction was determined to be:  $(k_H/k_D)_{\text{intra}} = 1.07 \pm 0.02$ .<sup>10</sup> The intermolecular

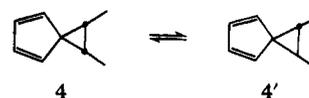
(2) S. F. Birch, R. A. Dean, N. J. Hunter, and E. W. Whitehead, *J. Org. Chem.*, 20, 1178 (1955).

(3) The reaction was carried out in sealed tubes, both in the gas phase and as a dilute benzene or pentane solution.

(4) Rate constants were determined by glc using benzene as solvent and pentane as internal standard. Least-squares analyses were performed to obtain the best fit of the data.

(5) For a review of the vinylcyclopropane rearrangement, see: C. D. Gutsche and D. Redmore, "Carbocyclic Ring Expansion Reactions," Academic Press, New York, N.Y., 1968, p 163.

(6) These values may be compared with the values of  $E_a = 42.7$  and  $\log A = 14.76$  for the geometrical isomerization of spiroheptadiene (4).<sup>7,8</sup>



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(10) This value is an average derived from five independent determinations, with the error being expressed as standard deviation.