Conformational Aspects of Muramic Acids. Analysis Based on Circular Dichroism Measurements*

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ABSTRACT: The circular dichroism spectra of muramic acid and some related compounds have been analyzed. Muramic acid has a weak positive ellipticity band at 244 mμ and a more intense negative band at 208 mμ. This spectrum is similar to that of D-lactic acid, suggesting that the lactyl moiety of muramic acid is of the D configuration, and that this moiety plays a prominent role in determining the overall shape of the circular dichroism spectrum. It appears that the O-3 atom of the 2-amino-2-deoxy-D-glucose moiety of muramic acid and the double bond of the carboxyl group exist in an eclipsed conformation.

The circular dichroism spectrum of *N*-acetylmuramic acid also features two overlapping ellipticity bands of opposite sign. An accurate approximation of the dichroic properties of *N*-acetylmuramic acid is obtained from the sum of the spectra of 2-acetamido-2-deoxy-D-glucose plus muramic acid, suggesting a close conformational identity among these compounds.

N-Acetylmuramic acid [2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose] is an integral component of the peptidoglycan units of bacterial cell walls (Salton, 1964; Ghuysen *et al.*, 1968). The absolute configuration of the lactyl residue on the 2-amino-2-deoxy-D-glucose moiety of muramic acid [2-amino-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose] was assumed to be D on the basis of $[\alpha]_D$ values (Strange and Kent, 1959), and recently additional evidence has been presented confirming this original conclusion (Matsushima and Park, 1962; Osawa and Jeanloz, 1965; Tipper, 1968; Veyriéres and Jeanloz, 1969). Conformational studies on muramic acid derivatives have been limited (Blix and Jeanloz, 1968) although the conformational features of the *N*-acetylmuramic acid residues in polysaccharides must be considered as a primary factor in determining the 3-dimensional structure of the peptidoglycan network, and in its interaction with lysozyme molecules (Phillips, 1967; Ghuysen, 1968; Chipman and Sharon, 1969).

Circular dichroism methods have been employed to study the conformation of acetamido sugars (Kabat *et al.*, 1969; Stone, 1969) and uronic acids (Listowsky *et al.*, 1969). In addition, rules relating the optical rotatory power of carboxylic acids to their conformation in solution have recently been developed (Listowsky *et al.*, 1970). Circular dichroism also offers a convenient means of isolating and studying the optical activity induced by the asymmetric environment in close proximity to a specific chromophore. In the present study, the circular dichroism spectra of muramic and *N*-acetylmuramic acids have been examined and compared with the spectra of related compounds. On analysis of the data obtained and by application of previously established principles, some conformational features of muramic acid have been revealed.

Experimental Section

Materials. Muramic acid was obtained from Sigma Chemical Co. (mp 136°, $[\alpha]_D^{25}$ + 126), and gave a single spot on paper chromatograms in 3 different solvent systems. The isomuramic acid [2-amino-3-*O*-(L-1-carboxyethyl)-2-deoxy-D-glucose] was generously provided by Drs. J. T. Park and R. D. Shaw of Tufts University School of Medicine (Matsushima and Park, 1962). This material was found to contain some inorganic substances and near-ultraviolet-absorbing materials. Most of the contaminants were removed by several recrystallizations and the purity of the sample used was estimated to be 75–80% (mp 127–135°) on the basis of the dry weight of the material and comparison with the molar extinction coefficients of muramic acid at 210 mμ. It was considered unlikely that the impurities had an appreciable effect on the observed circular dichroism spectrum, since the ratio between the absorption at 210 mμ and the molecular ellipticity remained constant after each recrystallization. *N*-Acetylmuramic acid was obtained from Sigma Chemical Co. and estimated to be 92% pure ($[\alpha]_D^{25}$ +44°, mp 120–123°). The other compounds were obtained from commercial sources, and were checked for optical purity by measurement of their $[\alpha]_D$ values.

Methods. All samples for the circular dichroism studies were dissolved in aqueous media, and the pH was adjusted by the addition of small amounts of HCl or NaOH. Circular dichroism measurements were made using a Cary Model 60 spectropolarimeter with a 6001 CD attachment. The instrument records the angle of ellipticity, θ, in degrees, and the data are reported in terms of molecular ellipticity, $[\theta]$, in deg cm²/dmole. The slit widths were programmed so that the spectral band width of the monochromator was 1.5 mμ or less over the entire spectral range.

A strain-free cylindrical cell with a path length of 1.0 cm was employed for all of the studies. Solution concentrations

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TABLE I: Circular Dichroism Data of Muramic Acid and Related Compounds.

Compound	Ellipticity Extrema: Wavelength (mμ)	Ellipticity Extrema: $[\theta]_\lambda$
Muramic acid		
pH 2.5	275; 244; 208	−14; 40; −5,200
pH 6.5	275; 225; 201	−5; 260; −4,700
Isomuramic acid		
pH 2.5	*a*; 207	*a*; 1800
pH 6.5	221; 203	−250; 800
D-Lactic acid		
pH 2.5	244; 210	18; −2900
pH 6.5	215	−700
D-Glucosamine		
pH 2.5	200[b]	−290
pH 6.5	200	−130
N-Acetylmuramic acid		
pH 2.5	245; 209	49; −12,600
pH 6.5	204	−15,500
N-Acetyl-D-glucosamine		
pH 2.5	211	−5,900
pH 6.5	211	−6,300

[a] Limited amounts of material precluded accurate ellipticity measurements at the longer wavelengths. [b] The ellipticity extrema were centered below 190 mμ, and were not accessible to the present instrumentation.

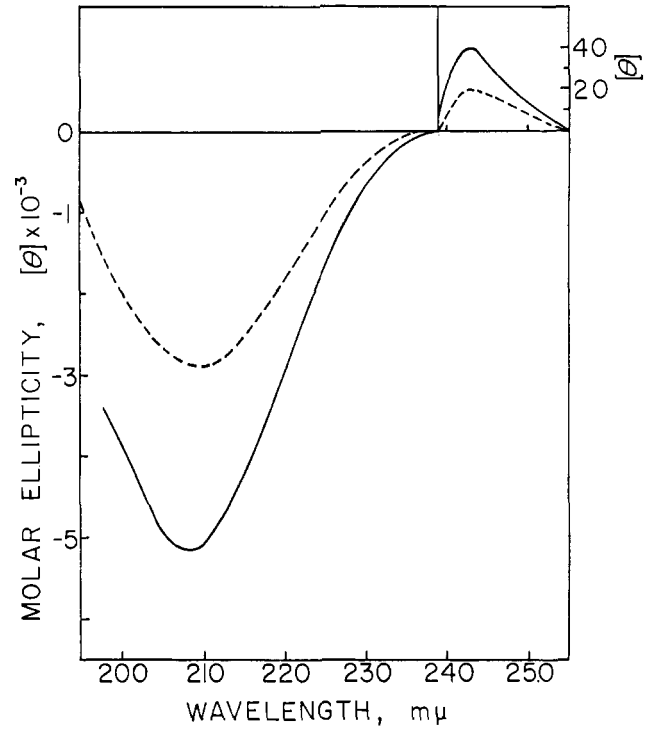

FIGURE 1: Circular dichroism spectra of muramic acid (solid line) and D-lactic acid (dotted line) in aqueous solution, pH 2.5. Measurements above 235 mμ were made with 1.0% solutions, and measurements below this wavelength were made with 0.1% solutions.

were adjusted to maintain absorption values of less than 2.0 over the entire spectral range. The solution temperatures were 25–27°, the temperature of the cell compartment. Each reported measurement was duplicated on at least three independent sample preparations.

Results

At pH 2.5, the circular dichroism spectrum of muramic acid is characterized by a negative ellipticity band at 208 mμ and a positive band of low intensity at 244 mμ (Figure 1). The distinct non-Gaussian appearance of the 244-mμ band is indicative of an appreciable overlap with the more intense shorter wavelength band. Therefore, the observed position of the low-intensity positive band does not define accurately the transition wavelength (Wellman *et al.*, 1965; Listowsky *et al.*, 1969). Accordingly, the actual energy difference between the two ellipticity extrema is probably much less than that observed.

The circular dichroism spectrum of muramic acid is similar to the spectrum of D-lactic acid (Figure 1) but both the 208-mμ and 244-mμ ellipticity bands of muramic acid are of greater intensity. Optically active absorption bands in this spectral region have previously been ascribed to the carboxyl groups and arising from the n–π^* transition (Barth *et al.*, 1969; Listowsky *et al.*, 1970). The other functional groups of muramic acid are expected to have their lowest energy absorption bands in the vacuum ultraviolet region. Indeed 2-amino-2-deoxy-D-glucose exhibits a very low degree of ellipticity above 200 mμ (Table I). A third very weak extremum centered near 275 mμ is also observed in the circular dichroism spectrum of muramic acid (Table I) and probably indicates the presence of small amounts of the acyclic form. A detailed account of the optical activity associated with open-chain forms of sugars of this type will be presented by us in a future report.

The ellipticity band at 208 mμ in the circular dichroism spectrum of isomuramic acid (Table I) is of similar shape but opposite in sign and of lower magnitude than the corresponding band of muramic acid. At neutral pH, muramic and isomuramic acids probably exist as zwitterions in solution (Strange and Kent, 1959; Kent and Strange, 1962) and their circular dichroism spectra are complex (Table I). Since theoretical considerations, relating the optically active transitions of carboxylate ions to their 3-dimensional structure have not been fully developed, these ionized forms will not be considered in the present conformational analyses.

Figure 2 shows the circular dichroism spectrum of *N*-acetylmuramic acid and the sum of the ellipticities of its constituents. The reducing sugars have been employed in this study since it has been shown that in aqueous solution, the effect of the anomeric disposition on the circular dichroism properties of 2-acetamido-2-deoxy sugars is relatively small (Kabat *et al.*, 1969; Listowsky and Englard, 1968). The curve obtained by summation of the circular dichroism spectra of *N*-acetyl-D-glucosamine (2-acetamido-2-deoxy-D-glucose) and D-lactic acid (curve 3) approximates the curve of the *N*-acetylmuramic acid spectrum. The sum of the circular dichroism spectra of *N*-acetyl-D-glucosamine plus muramic acid (curve 2) also gives a curve that is almost identical with that obtained with *N*-acetylmuramic acid. On the other hand,

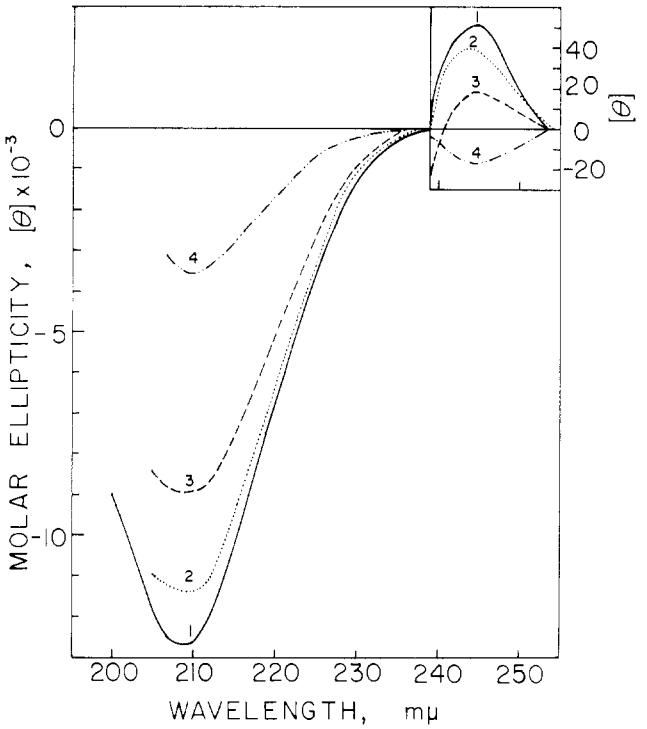

FIGURE 2: Circular dichroism spectrum of *N*-acetylmuramic acid (curve 1) and the molecular ellipticities of *N*-acetylglucosamine plus muramic acid (curve 2), *N*-acetylglucosamine plus D-lactic acid (curve 3), and *N*-acetylglucosamine plus L-lactic acid (curve 4). Concentrations were analogous to those indicated in Figure 1.

compared with the circular dichroism spectra of *N*-acetylmuramic acid, the curve obtained from the sum of the circular dichroism spectra of *N*-acetyl-D-glucosamine plus L-lactic acid reveals a longer wavelength band which is of opposite sign and a short-wavelength band which is of much lower intensity (curve 4). It is evident from the data presented in Figure 2, that the longer wavelength band of *N*-acetylmuramic acid reflects the contribution of the carboxyl group exclusively, whereas the shorter wavelength band is associated with the electronic transitions of both the amide and carboxyl chromophores.

Discussion

The circular dichroism spectrum of muramic acid, with a negative ellipticity band at 208 mμ and positive band near 244, resembles the spectrum of D-lactic acid (Figure 1) and the spectra of other α-hydroxy and alkoxy acids in the D configuration (Listowsky *et al.*, 1970). In addition, a spectrum similar to that of *N*-acetylmuramic acid may be obtained by the summation of the spectra of *N*-acetyl-D-glucosamine and D-lactic acid, whereas there is a wide disparity if the comparison is made to the sum of the spectra of *N*-acetyl-D-glucosamine and L-lactic acids. These results suggest that the carboxyethyl moiety of muramic acid is in the D configuration, and substantiate previous conclusions based on the stereoselective synthesis of muramic acid (Matsushima and Park, 1962; Osawa and Jeanloz, 1965), on the enzymatic determination of the liberated lactic acid obtained on alkaline degradation of muramic acid (Tipper, 1968; Wheat *et al.*, 1969), and on the recent structural proof by Veyriéres and Jeanloz (1969). It is also evident from the present study that the muramic acid dichroic bands are associated with the optically active transitions of the carboxyl group, and their shapes are determined by the disymmetric perturbation of this chromophore. The molecular environment in close proximity to the carboxyl group, and particularly the stereochemistry of the α-carbon atom, thus play a significant role in determining the characteristics of the circular dichroism spectrum of muramic acid.

It had been shown that circular dichroism measurements may be used to study rotational isomerism about sp^3–sp^2 carbon–carbon bonds of carboxylic acids, and to predict preferred conformations (Listowsky *et al.*, 1970). Conformational equilibria have been proposed (Barth *et al.*, 1969; Listowsky *et al.*, 1970) to explain the presence of multiple overlapping ellipticity bands such as those observed for muramic and *N*-acetylmuramic acids. For the simple α-hydroxy and α-alkoxy acids, such as lactic acid and its derivatives, the carboxyl group and an α-carbon substituent were assumed to be coplanar. Moreover, for these compounds, structures with the α-carbon substituent and the double bond of the carboxyl group in an eclipsed conformation have been designated as the energetically favored forms (Listowsky *et al.*, 1970). The relative proportion of this rotational isomer can be altered by adjusting environmental conditions such as solvent polarity or temperature, but this form generally predominates unless precluded by inherent steric factors. If these considerations are similarly applied in the present study, the favored rotamer of muramic acid in aqueous solution will have the O-3 atom of the 2-amino-2-deoxy-D-glucose moiety and the double bond of the carboxyl group in an eclipsed conformation. The presence of the longer wavelength band at 244 mμ in the circular dichroism spectrum of muramic acid suggests that this form is in equilibrium with another less favored rotational isomer (probably having eclipsed carbonyl and methyl groups).

The differences in magnitude of ellipticity of muramic acid as opposed to D-lactic acid (Figure 1) is probably due to certain specific conformational features of the muramic acid molecule. Thus, the conformation of the carboxymethyl group may deviate from the aforementioned eclipsed form, or a change may have occurred in the perturbation of the carboxyl chromophore induced by the spatial alignment of the D-glucosamine ring about the C-3 ether linkage. Although the former explanation cannot be ruled out entirely, the latter interpretation is more reasonable on the basis of the data obtained with isomuramic acid. Thus, the 208-mμ negative band of muramic acid is approximately 2.5 times the intensity of the 208-mμ positive band of isomuramic acid. Also, in contrast to muramic acid when compared with D-lactic acid (Figure 2), the magnitude of ellipticity of isomuramic acid is appreciably less than that of L-lactic acid. By assuming a negative rotational strength associated with the influence of the ring disposition, one can explain the difference in the magnitude of the 208-mμ bands of muramic and isomuramic acids and also account for the differences between these acids and the corresponding isomers of lactic acid. The D-glucosamine moiety probably assumes an energetically favorable orientation about the O-3 ether bond that is independent of the configuration of the α-carbon atom of the lactic acid. It is thus proposed that the alignment of the D-glucosamine moiety with respect to the carboxyl group is identical for both

muramic and isomuramic acids, and contributes negative ellipticity for both. The exact spatial disposition of the ring relative to the carboxyl group cannot as yet be defined, and must await the completion of rigorous quantitative treatments of the optically active transitions of carboxyl groups.

As a result of rotational freedom about the C-3 ether linkage, the muramic acid and *N*-acetylmuramic acid molecules may exhibit appreciable conformational flexibility. Conformations with the carboxyl and C-2 amino group in close spatial juxtaposition are required for the ring closure to form the internal amide (Carroll, 1963), but other conformations with the carboxyl and C-2 substituent far removed from each other are also conceivable and may actually be energetically preferred. The data in Figure 2 show that the sum of the circular dichroism spectra of *N*-acetyl-D-glucosamine and muramic acid gives a curve that closely approximates the spectrum of *N*-acetylmuramic acid. Ostensibly, therefore, the carboxyl and amide chromophores of *N*-acetylmuramic acid behave independently, and there are no interactions between them that would give rise to perturbations of their individual transition moments (Schellman and Nielsen, 1967). It is also evident that, in contrast to the numerous interactions between the asymmetric centers encountered in an optical rotatory dispersion analysis of acetamido sugars (Listowsky *et al.*, 1968), circular dichroism measurements can isolate the optical activity associated with the local environment about the carboxyl and amide chromophores. Since the asymmetry induced in the amide transitions arises from the stereochemistry at C-2 of the 2-acetamido-2-deoxy sugar, and circular dichroism can clearly differentiate between axial and equatorial acetamido substituents at C-2 (Listowsky *et al.*, 1968), it follows that the ring conformations of *N*-acetyl-D-glucosamine, muramic, and *N*-acetylmuramic acid must be almost identical (in all probability the C-1 conformation).

The results of the present study are in accordance with the suggestion that the spatial orientation of the acetamido substituent of *N*-acetylmuramic acid is very similar to its alignment in *N*-acetyl-D-glucosamine which does not contain a carboxyl group. For the latter compound, structures in which the amide group and C-5 of the ring are coplanar, and the amide carbonyl and the C-2 hydrogen are in a *cis* relationship have been proposed on the basis of optical rotatory dispersion studies (Beychock and Kabat, 1965) and for the lysozyme enzyme substrate complex (Phillips, 1967). From the data obtained it is also evident that *N*-acetylmuramic and muramic acids are conformationally similar. Probably, the O-3 atom and carbonyl of the carboxyl groups are in an eclipsed conformation, and the *N*-acetyl-D-glucosamine and D-glucosamine rings are in the same spatial disposition relative to their respective carboxyl groups. With these steric limitations, the likelihood of interactions between the carboxyl and amide groups of *N*-acetylmuramic acid *via* systems such as 8- or 9-member asymmetrically hydrogen-bonded rings, becomes rather remote.

References

Barth, G., Voelter, W., Bunnenberg, E., and Djerassi, C. (1969), *Chem. Commun.*, 355.

Beychok, S., and Kabat, E. A. (1965), *Biochemistry 4*, 2565.

Blix, G., and Jeanloz, R. W. (1969), *Amino Sugars 1*, 213.

Carroll, P. M. (1963), *Nature 197*, 694.

Chipman, D. M., and Sharon, N. (1969), *Science 165*, 454.

Ghuysen, J. M. (1968), *Bact. Rev. 32*, 425.

Ghuysen, J. M., Strominger, J. L., and Tipper, D. J. (1968), *Compr. Biochem. 26*, 53.

Kabat, E. A., Lloyd, K. O., and Beychok, S. (1969), *Biochemistry 8*, 747.

Kent, L. H., and Strange, R. E. (1962), *Methods Carbohyd. Chem. 1*, 750.

Listowsky, I., Avigad, G., and Englard, S. (1968), *Carbohyd. Res. 8*, 205.

Listowsky, I., Avigad, G., and Englard, S. (1970), *J. Org. Chem. 35*, 1080.

Listowsky, I., and Englard, S. (1968), *Biochem. Biophys. Res. Commun. 30*, 329.

Listowsky, I., Englard, S., and Avigad, G. (1969), *Biochemistry 8*, 1781.

Matsushima, Y., and Park, J. T. (1962), *J. Org. Chem. 27*, 3581.

Osawa, T., and Jeanloz, R. W. (1965), *J. Org. Chem. 30*, 448.

Phillips, D. C. (1967), *Proc. Nat. Acad. Sci. U. S. 57*, 484.

Salton, M. R. J. (1964), The Bacterial Cell Wall, Elsevier, Amsterdam.

Schellman, J. A., and Nielsen, E. B. (1967), *Conform. Biopolym. Pap. Int. Symp.*, 109.

Stone, A. L. (1969), *Biopolymers 7*, 173.

Strange, R. E., and Kent, L. H. (1959), *Biochem. J. 71*, 333.

Tipper, D. J. (1968), *Biochemistry 7*, 1441.

Veyriéres, A., and Jeanloz, R. W. (1969), 158th National Meeting of the American Chemical Society, New York, N. Y., Carb 17.

Wellman, K. W., Laur, P. H. A., Briggs, W. S., Moscowitz, A., and Djerassi, C. (1965), *J. Amer. Chem. Soc. 87*, 66.

Wheat, R. W., Kulkarni, S., Cosmatos, A., Scheer, E. R., and Steele, R. S. (1969), *J. Biol. Chem. 244*, 4921.