isomers and some other indigoids of special interest we hope to report in due time.

Ancient crushed shells, excavated from a former Phoenician dye industry near Sidon at the Lebanese coast, were identified as belonging to Murex Trunculus, a mollusk quite common at the rocky shores of Lebanon. Natural Tyrian Purple was extracted from freshly caught animals, following instructions given by Aristotle[4] and Pliny.[5] The e.s.r. spectrum obtained is typical for indigo and was found to be identical to the one of 6,6′-dibromoindigo, kindly synthesized for us by Dr. Floyd Tyson of Philadelphia. Earlier it was shown by Friedlaender[6] that juices extracted from Murex Brandaris, a species found mainly in the northern Mediterranean, contain 6,6′-dibromoindigo as the principal pigment. Our analysis gives final proof that also the old Phoenician dye consisted mainly of 6,6′-dibromoindigo and that it is justified to call the latter compound "Tyrian Purple."

Conclusions

The e.s.r. spectra of the basic indigoid dyes could all be fully explained by assuming that there are four successive triplet splittings, with splitting constants ranging from about 2 gauss to about 0.5 gauss, due to the four pairs of aromatic ring protons, and additional quintets if the heteroatoms are nitrogens. In the earlier communication on thioindigo[3] it was shown how each of these triplets should be assigned to a particular proton pair. The similarity of the spectra justifies the adoption of the same assignment for the cases that the hetero-atoms are selenium, oxygen, or nitrogen. The effect of the heteroatom substitution is mainly one of over-all increase of odd electron density in the outer rings of the molecule, about proportional to the electronegativity[7] of the Z-atoms. As a result the splitting constants in the molecule with Z-atoms of large electron affinity will be larger and produce wider e.s.r. spectra. The splitting constants in gauss for the various aromatic ring proton pairs, as resulting from our analysis, are collected in Table I. The error in these data is about 5%. Note that the splitting constants for selenium, sulfur, and nitrogen are obtained with acetone solutions, whereas for oxygen glacial acetic acid had to be used.

In the series Z = Se, S, O, N the increase in the observable over-all odd-electron density must be at the expense of the density in part (namely the chain O=C—C—C—C—O⁻) of the central H-shaped chromophore.[8]

Acknowledgment.—This investigation is sponsored by the U. S. Research Corporation, the National Science Foundation, and the Rockefeller Fund of the School of Arts and Sciences of our University. Several firms (BASF, Ciba, General Dyestuff, Hoechst, ICI, Light and Co.) most generously supplied indigoid derivatives. The authors thank Dr. W. Luettke for clarifying discussions and for samples of selenoindigo and oxindigo.

(4) Aristoteles, "Historia animalium," Book 4, Athens, -0340.
(5) C. Plinius, "Historia naturalis," Book 9, chap. 60–64, Roma, 0075.
(6) P. Friedlaender, *Monatsh. Chem.*, **28**, 991 (1907); **30**, 247 (1909).
(7) C. A. Coulson, "Valence," Oxford University Press, New York, N. Y., 1951, chap. 5, Table 10.
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An N.m.r. Study of the Glycoside Link in Glycosides of Glucose and Galactose[1a,b]

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Received August 24, 1962

Whether the glycosidic link in disaccharides is α or β can be determined from Hudson's rules[2,3] or enzymatic hydrolysis studies.[4] In this paper we will show how proton n.m.r. spectroscopy may be used to determine link configurations in glycosides of glucose and galactose.

Fig. 1 shows the n.m.r. spectra of glucose (I),

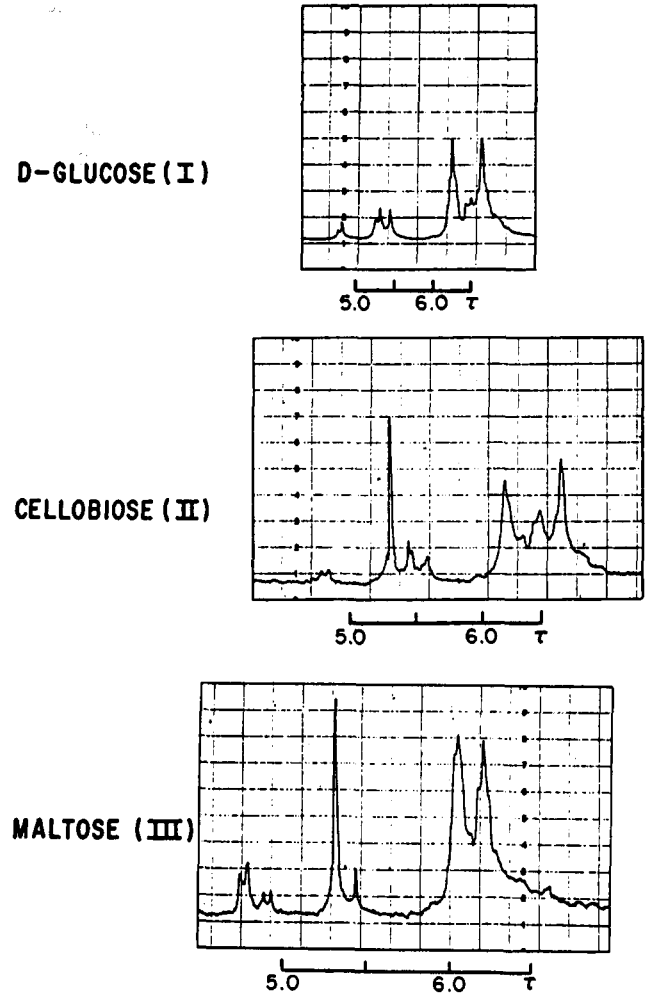


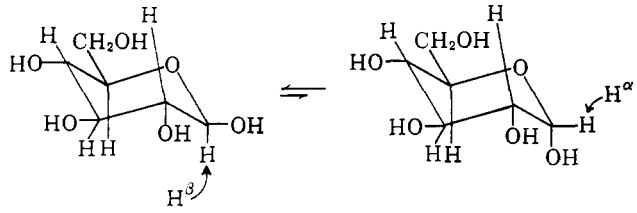
Fig. 1.—N.m.r. spectra of glucose derivatives at 60 Mc./sec. (ref. DSS).

cellobiose (II), and maltose (III) at 60 Mc. in deuterium oxide solution. The abscissa is in τ values[5] with the reference being the $(CH_3)_3Si$— line of sodium 2,2-

(1) (a) This investigation was supported by Public Health Service research grants B-904 and B-3370 from the National Institute of Neurological Diseases and Blindness, U. S. Public Health Service. (b) Present address: Stevens Institute of Technology, Hoboken, N. J.
(2) C. S. Hudson, *J. Am. Chem. Soc.*, **31**, 66 (1909).
(3) C. S. Hudson, *ibid.*, **60**, 1537 (1938).
(4) H. Baumann and W. Pigman, "The Carbohydrates," W. Pigman, ed., Academic Press, Inc., New York, N. Y., 1957, chap. X.
(5) G. V. D. Tiers, *J. Phys. Chem.*, **62**, 1151 (1958).

dimethyl-2-silapentane-5-sulfonate (hereinafter DSS), a water-soluble internal reference compound whose main resonance is at the same frequency as the commonly used n.m.r. reference tetramethylsilane.[6,7] The position of the anomeric proton on C-1 is unique and appears at low applied magnetic field[8] (4.58 to 5.58 τ) because the carbon to which it is attached bears two electron-withdrawing oxygen atoms. All other protons are attached either to oxygen or to a carbon bearing at most one oxygen atom. Thus the resonance of the protons attached to C-2, C-3, C-4, C-5, and C-6 fall in the region 5.67 to 6.67 τ and, although the spectrum in this region is characteristic for the particular sugar moiety, this part of the spectrum will not be analyzed in the present paper. The protons attached to oxygen form an exchanging pool with the residual water in the deuterium oxide solution and have a single sharp resonance near 5.25 τ.

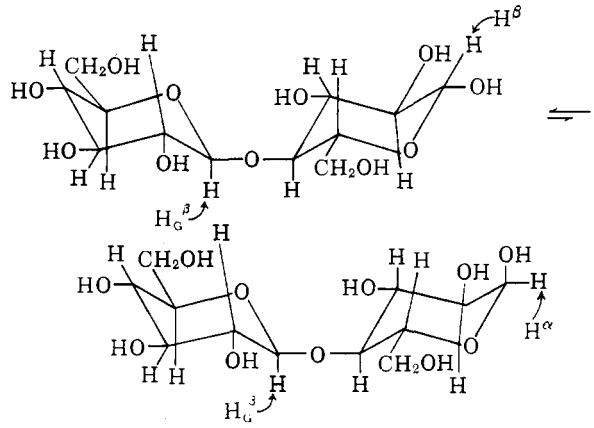
The spectrum of D-glucose (I) illustrates the effect of mutarotation on the resonance of the anomeric proton in a mono- or a reducing oligosaccharide. The ratio of β to α is roughly 2:1[9]; thus the doublet at



I (β-D-glucose) I (α-D-glucose)

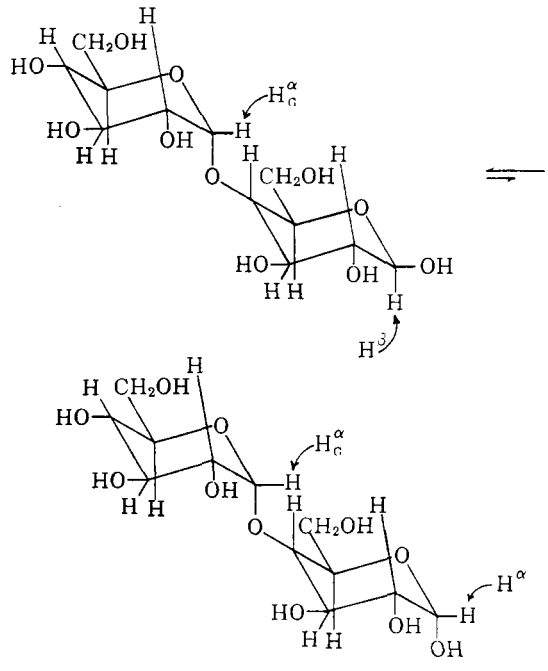
5.35 τ must be due to H^β while the doublet at 4.78 τ is due to H^α (H^β and H^α are the anomeric protons in β- and α-D-glucose, respectively). The assignment of line positions is in accord with other considerations since an axially oriented hydrogen (H^β) is more shielded than an equatorially oriented hydrogen (H^α) and should resonate at a higher applied magnetic field.[10] Furthermore, the spin–spin coupling constant between the protons on C-1 and C-2 is 3.0 c.p.s. in the case of α-D-glucose (typical of axial–equatorial couplings[10]), and 7.4 c.p.s. for β-D-glucose (typical of axial–axial coupling[10]).

In 4-O-β-D-glucopyranosyl-D-glucose (cellobiose) (II) the anomeric proton at the glycosidic link (H_G^β) resonates at 5.50 τ with a J_{12} coupling constant of 7.4 c.p.s. In the spectrum there appear the two doublets already observed in the glucose spectrum for H^β, 5.35 τ (J_{12} = 7.4 c.p.s.), and for H^α, 4.78 τ (J_{12} = 3.4 c.p.s.). Maltose (III) 4-O-α-D-glucopyranosyl-D-glucose) illustrates an α glycosidic link in which the anomeric proton at the glycosidic link (H_G^α) resonates at 4.62 τ (J_{12}= 3.4 c.p.s.) which is near that observed for H^α (4.80 τ, J_{12} = 3.4 c.p.s.) in the same spectrum. The two



II (Cellobiose)

cannot be confused since H_G^α is a more intense doublet than H^α. Every molecule of maltose has an H_G^α proton while only part of the molecules have an H^α proton. In addition, maltose has a doublet due to H^β (5.37 τ, J_{12} = 7.4 c.p.s.). In both maltose and



III (Maltose)

cellobiose the position, intensity, and coupling constants for H^α and H^β are quite near those observed for D-glucose. A possible conformation for maltose with the glycosidic link inverted seems unlikely, as it would place four groups in the nonreducing portion of the disaccharide in a sterically hindered axial position as opposed to only one axial group in conformation III. Furthermore, the observed chemical shift of 4.62 τ is far removed from that observed for axial anomeric hydrogen in glucose and cellobiose. Similar arguments can be applied to show cellobiose really has the conformation II and not one with an equatorial anomeric hydrogen since the latter would require an equatorial–equatorial coupling constant of 3 c.p.s. instead of the observed 7 c.p.s.[10] Although II and III undoubtedly show the true conformation of the glycosidic link in maltose and cellobiose the possibility of doubt with some other monosaccharides leads us to restrict the present study to glycosides and oligosaccharide of

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(7) G. V. D. Tiers and A. Kowalsky, Abstract Papers, 137th National Meeting of the American Chemical Society, Cleveland, Ohio, 1960, p. 17-R.

(8) All line positions are measured to the center of the doublets.

(9) H. S. Isbell and W. W. Pigman, *J. Res. Natl. Bur. Std.*, **18**, 141 (1937).

(10) R. U. Lemieux, R. K. Kullnig, H. J. Bernstein, and W. G. Schneider, *J. Am. Chem. Soc.*, **79**, 1005 (1957); **80**, 6098 (1958); **82**, 6427 (1960); R. U. Lemieux, R. K. Kullnig, and R. Y. Moir, *ibid.*, **80**, 2237 (1958); S. Brownstein, *ibid.*, **81**, 1606 (1959); J. I. Musher, *J. Chem. Phys.*, **34**, 594 (1961); M. Karplus, *ibid.*, **30**, 11 (1959); J. I. Musher, *J. Am. Chem. Soc.*, **83**, 1146 (1961); S. Brownstein and R. Miller, *J. Org. Chem.*, **24**, 1886 (1959); R. W. Lenz and J. P. Heeschen, *J. Polymer Sci.*, **51**, 247 (1961).

TABLE I

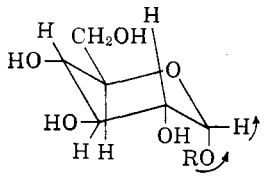
N.M.R. SPECTRA OF MONO- AND OLIGOSACCHARIDES[a] IN D_2O

	H_G^β	H^β	H^α	H_G^α
D-Glucose		5.37[b] (7.4)[c]	4.78 (3.0)	
4-O-α-D-Glucopyranosyl-D-glucose (maltose)		5.37 (7.4)	4.80 (3.4)	4.62 (3.4)
4-O-β-D-Glucopyranosyl-D-glucose (cellobiose)	5.50 (7.0)	5.35 (7.4)	4.78 (3.3)	
6-O-β-D-Glucopyranosyl-D-glucose (gentiobiose)	5.50 (7.7)	5.34 (7.6)	4.78 (3.5)	
α-D-Glucopyranosyl-β-D-fructofuranoside (sucrose)				4.59 (3.2)
1-O-α-D-Glucopyranosyl-α-D-glucose (α, α-trehalose)				4.81 (3.2)
3-O-α-D-Glucopyranosyl-D-fructose (turanose)				4.70 (3.2)
3-O-Methyl-D-glucose		5.37 (6.2)	4.81 (2.3)	
1-O-α-Methyl-D-glucoside				5.21 (3.0)
1-O-β-Methyl-D-glucoside	5.62 (7.4)			
D-Galactose		5.43 (6.7)	4.77 (2.7)	
4-O-β-D-Galactopyranosyl-D-glucose (lactose)	5.58 (7.1)	5.36 (7.3)	4.78 (3.7)	
6-O-α-D-Galactopyranosyl-D-glucose (melibiose)		5.33 (7.7)	4.79 (2.9)	5.04 (2.9)
O-α-D-Glucopyranosyl-(1 → 3)-O-β-D-fructofuranosyl-				4.54 (3.1)
(2 → 1)-α-D-glucopyranoside (melezitose)				4.81 (3.2)
O-α-D-Galactopyranosyl-(1 → 6)-O-α-D-glucopyranosyl-				5.02 (2.7)
(1 → 2)-β-D-fructofuranoside (raffinose)				4.59 (2.9)
Sodium D-glucuronate		5.37 (8.1)	4.77 (3.4)	
D-Glucuronic acid		5.28 (8.8)	4.73 (2.8)	
D-Galacturonic acid		5.38 (7.8)	4.66 (3.2)	
D-Glucosamine hydrochloride		5.04 (8.5)	4.54 (3.8)	
N-Acetyl-D-glucosamine			4.81 (2.7)	
D-Galactosamine hydrochloride		5.12 (8.3)	4.53 (3.8)	

[a] Line positions in τ values. [b] Chemical shifts to center of doublet (accurate to ±0.01 p.p.m.). [c] Number in parentheses is the J_{12} coupling constant in c.p.s. (accurate to ±0.3 c.p.s.).

glucose and galactose where a change in conformation about the glycosidic link would lead to a large unfavorable axial to equatorial ratio. With this restriction we find an amazing correlation between line positions, coupling constants, and whether a proton is classified as H^α, H^β, H_G^α, or H_G^β.

The upper part of Table I summarizes our data for the n.m.r. spectra of a number of mono-, di-, and trisaccharides between 5.67 and 4.58 τ. In reducing sugars H^β (the hydroxyl at the anomeric carbon is β) resonates at 5.37 ± 0.05 τ with J_{12} = 7.0 ± 0.7 c.p.s. This coupling constant is quite close to that observed for axial–axial coupling in acetylated monosaccharides and in cyclohexane systems.[10] H^α (the hydroxyl at the anomeric carbon is α) resonates at 4.78 ± 0.01 τ with a coupling constant J_{12} = 3.2 ± 0.5 c.p.s., which is quite close to the coupling constants observed for axial–equatorial or equatorial–equatorial coupling. The glycosidic anomeric proton absorption H_G^β occurs at 5.56 ± 0.06 τ (J_{12} = 7.2 ± 0.2 c.p.s.) while that for H_G^α is much more variable, namely, 4.88 ± 0.33 τ (J_{12} = 3.2 ± 0.6 c.p.s.). This variation may be due to varying 1–3 steric interaction of the —OR group distorting the six-membered ring so as to force H_G^α somewhat out of its normal equatorial environment. It might be thought that absorption as high as 5.22 τ



(as observed for α-methyl-D-glucoside) could be confused with H^β or H_G^β; however, the coupling constant of 3 c.p.s. for an α link as opposed to 7 c.p.s. for a β link will prevent misinterpretation of the spectrum.

In the lower part of Table I are listed some compounds related to glucose and galactose showing values of H^α, H^β, and J_{12} coupling constants very near those observed for the glycosides. It is not surprising that the chemical shifts of the last three compounds are different from the others since replacing an hydroxide on C-2 with either an amine hydrochloride or an N-acetylamino group should have a marked effect on the chemical shift of the adjacent anomeric proton. The J_{12} coupling constants are about what one would expect for the α and β isomers, however. In most cases where mutarotation is possible the H^β resonance is more intense than H^α absorption with the interesting exception of N-acetylglucosamine. Perhaps hydrogen bonding contributes to the stability of what would normally be regarded as the more unstable axial hydroxyl form. In any case, there is no indication for the presence of the β form in solution.

Experimental

The samples which were 10% sugar and 2% DSS were prepared by dissolving the sugar plus reference in 5 ml. of deuterium oxide, pumping away the deuterium oxide, dissolving the residue in 0.5 ml. of deuterium oxide, and sealing the resulting solution in 5 mm. n.m.r. tubes. In some cases enough HDO resonance line remained to obscure the fine structure of the doublet near 5.33 τ. In these cases the sugar was exchanged twice with 5-ml. portions of deuterium oxide so that the remaining HDO line did not interfere with the resolution of nearby doublets. The sugars were the highest purity available from Cal-Biochem and were used without further purification.

Spectra were taken on a Varian HR60 nuclear magnetic resonance spectrometer and were calibrated by audio side bands from a variable frequency oscillator whose frequency was checked by a cycle counter. Sufficient spectra were taken in each case (usually 8) so that the mean square error of the mean line position[11] was less than 0.01 p.p.m.[8] Coupling constants had a mean square error of the mean of less than 0.3 c.p.s.

Sodium 2,2-dimethyl-2-silapentane-5-sulfonate monohydrate (DSS) was prepared by the method of Tiers.[6]

(11) J. B. Scarborough, "Numerical Mathematical Analysis," The Johns Hopkins Press, 1958, p. 436.