Electron Paramagnetic Resonance Studies of Nitric Oxide Hemoglobin Derivatives. I. Human Hemoglobin Subunits*

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ABSTRACT: To elucidate the molecular conformation of a ligand bound to hemoglobin, nitric oxide derivatives of human adult hemoglobin and its subunits (α and β) have been studied by electron paramagnetic resonance spectroscopy.

The spectrum of hemoglobin α-NO and β-NO are dissimilar and that of erythrocytes NO and human adult hemoglobin NO are the arithmetic mean of the spectrum for the isolated subunits. At room temperatures, the signal of hemoglobin α-NO exhibits axial symmetry, whereas that of hemoglobin β-NO slightly deviates from axial symmetry. Below $-50°$, however, the signal for both the subunits markedly distorts from axial symmetry. The approximate principal g values have been estimated by comparing the observed spectra with simulated spectra calculated by a digital computer. For the simulation, a broad intrinsic line width had to be assumed, which may be due to a freedom of the orientation of NO on heme. Urea and high pH broaden the signal of hemoglobin β-NO. The effect of temperature and denaturing agents on the signal shape can be explained by the change of orientational freedom of liganded NO associated with the conformational change of globin moiety.

Since the method of isolating Hb α[1] and Hb β subunits from Hb A has been established (Bucci and Fronticelli, 1965; Tyuma *et al.*, 1966), differences in various physicochemical properties of the subunits and between the subunits and their native or reconstituted parent molecule have been demonstrated. These include oxygen affinity, absorption spectra (Antonini *et al.*, 1965; Tyuma *et al.*, 1966), and circular dichroism (Beychok *et al.*, 1967). Further accumulation of such data will be very useful for the understanding of physiological functions of hemoglobin at a molecular level. One of the approaches in this problem is an analysis of the electronic structure of the heme–ligand complex in hemoglobin as related to the conformation of its protein moiety. However, previous studies performed along this line did not explicitly disclose the electronic structure of the complex in the two subunits and in the parent molecule.

Although the electron paramagnetic resonance technique has been successfully applied to the study of the electronic structure of ferrihemoglobin derivatives, most of the physiologically important ferrohemoglobin derivatives, including oxyhemoglobin, exhibits no detectable electron paramagnetic resonance signal. However, it was shown that nitric oxide, similar to oxygen, binds to the sixth coordination position of the heme in ferrohemoglobin, giving a distinct electron paramagnetic resonance spectrum (Ingram and Bennett, 1955). Moreover, Griffith (1956) suggested a possibility of estimating the molecular orientation and electronic structure of the NO molecule on heme through an analysis of principal g values of the spectrum, and Gordy and Rexford (1961) showed that the shape of the spectrum depends upon the primary structure of the globin moiety of the hemoglobin. In a preliminary communication we also demonstrated the difference in electron paramagnetic resonance signals of NO derivatives of hemoglobin subunits at low temperatures (Shiga *et al.*, 1968).

In the present paper, therefore, a systematic study was made on the NO derivatives of erythrocytes, Hb A, Hb α, and Hb β by electron paramagnetic resonance spectroscopy to elucidate the molecular conformation of the heme–NO complex, especially the orientation of the NO molecule with respect to heme.

Experimental Section

Materials. Hb A was prepared as described by Benesch and Benesch (1962) from the blood of young, healthy, adult males. Hb α and Hb β were prepared according to the method of Tyuma *et al.* (1966), and are freed from *p*-mercuribenzoate. Unless otherwise stated, their 1–5% solutions in 0.1 M phosphate buffer at pH 7.4 were used for experiments. Concentrations of all solutions were determined by spectral analysis after conversion into the cyanomet derivatives at 540 mμ (ϵ 11.5 $\times$ 10^3/heme).

All the reagents were reagent grade and used without further purification. NO and CO gases were supplied from Takachiho Kagaku Co. and had a purity of 98.5 and 99.9%, respectively.

Apparatus. A Varian V-4500 electron paramagnetic resonance spectrometer with a 100-kc modulation unit and variable temperature accessory was employed. Microwave frequency was measured by a cavity frequency meter. Apparent g values and line width were calibrated from the electron paramagnetic resonance signals of

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[1] Abbreviation used: Hb α and Hb β, α and β chains of Hb A; Hb A, human adult hemoglobin.

diphenylpicrylhydrazyl and *N*-1-oxyl-2,2,6,6-tetramethyl-4-piperidinol inserted in or besides the sample.

Methods. The Hb NO derivatives were prepared as follows. Hb-O_2 solutions were first exposed to CO gas under atmospheric pressures and converted to Hb-CO completely, then the solutions were slightly evacuated and NO gas was introduced anaerobically up to 1 atm. A quartz tube (3-mm diameter) or flat cell (0.3 × 10 mm) of Thunberg tube type was employed. For variable temperature studies, the solutions were sealed anaerobically in a glass capillary (1-mm diameter). The completeness of NO equilibration was confirmed spectrophotometrically as described by Gibson and Roughton (1957a).

Estimation of g Values. Since the paramagnetic NO molecule is bound to macromolecules, its *g* tensors and hyperfine couplings are not averaged out. This led us to the use of the following simulation method for the estimation of *g* values (Lefevre and Maruani, 1965; Kuwata, 1967) with an assumption that NO molecules in a fixed orientation relative to heme give a signal with definite principal *g* values and anisotropic nitrogen hyperfine couplings (*A*'s). An NEC-NEAC 2203 digital computer was used for the calculation. First, a proposed set of principal *g* values was given for calculating the shape function. Then, the Gauss function of an appropriate intrinsic line width[2] or a set of *A* values with adequate intrinsic line width was given. Finally, the first derivative of the simulated spectrum was printed out. The process was repeated, varying the *g* values and the line widths, until the best-fit spectrum was obtained. The use of Gaussian rather than Lorentzian functions in this case has been recommended by Lefevre and Maruani (1965) as will be discussed later.

Unexpectedly, it was impossible to simulate exactly any one of the observed spectra from a given set of *g* and *A* values as can be seen in Figure 1, in which several simulated spectra of Hb α-NO at low temperatures are shown as an example. In addition, either Gauss function or nitrogen hyperfine couplings with the intrinsic line width of less than 30 gauss gave seriously unmatched spectra. As will be discussed later, these facts suggest that the observed spectrum does not arise from NO molecules in a fixed orientation, but a composite of signals with different *g* and *A* values. Therefore, the estimated principal *g* values described below are an approximate set within an error of ±0.003 in *g*.

Results

Room Temperature Spectra. Electron paramagnetic resonance spectra of NO derivatives of Hb A, Hb α, and Hb β at 19° are shown in Figure 2. A human erythrocyte suspension equilibrated with NO gave a spectrum similar to Hb A-NO. The spectra of the two subunit NO's are dissimilar, and the spectrum of Hb A-NO is the arithmetic mean of that for the isolated chains. In Hb A-NO, the apparent *g* value is 2.02, the width between maximal deflection points is about 70 gauss, and the line shape is nearly symmetric. These characteristics are in good agreement with those reported by Sancier *et al.* (1962). On the other hand, the spectra of Hb α-NO

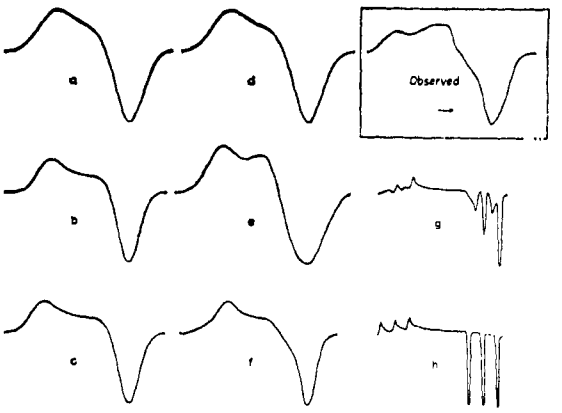

FIGURE 1: Comparison of observed and simulated electron paramagnetic resonance spectra of Hb α-NO. Observed spectrum was recorded at −130°. Modulation amplitude was 0.8 gauss. Span of arrow corresponds to 20 gauss and vertical line shows the position of the diphenylpicrylhydrazyl signal. *g* values and intrinsic line width introduced into the calculation were as follows: (a) g = 2.000, 2.000, and 2.074; Gauss function of 42 gauss width. (b) g = 2.000, 2.000, and 2.080; Gauss function of 42 gauss width. (c) g = 2.000, 2.000, and 2.085; Gauss function of 42 gauss width. (d) g = 2.000, 2.000, and 2.080; Gauss function of 56 gauss width. (e) g = 1.987, 2.000, and 2.074; Gauss function of 56 gauss width. (f) g = 2.000, 2.000, and 2.080; nitrogen hyperfine couplings = 10, 10, and 20 gauss with line width of 28 gauss. (g) g = 2.000, 2.000, and 2.080; nitrogen hyperfine couplings = 10, 10, and 20 gauss with line width of 4 gauss. (h) g = 2.000, 2.000, and 2.080; nitrogen hyperfine couplings = 20, 20, and 20 gauss with line width of 4 gauss.

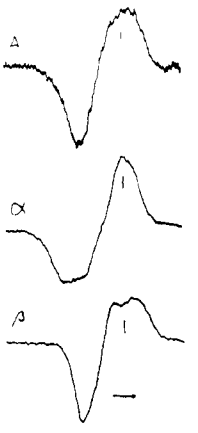

FIGURE 2: Electron paramagnetic resonance spectra of NO derivatives of Hb A, Hb α, and Hb β at 19°. Modulation amplitude, 0.8 gauss; time constant, 0.3 sec. Span of arrow corresponds to 33.2 gauss and vertical line shows the position of the diphenylpicrylhydrazyl signal.

[2] To avoid confusion, following terms are used to express the three kinds of "line width." (a) "Apparent line width" is the "width between maximal deflections" of the observed first derivative spectrum. (b) The real line width of the component hyperfine line or Gauss function, which cannot be determined experimentally, is expressed as "component line width." (c) The proposed line width of the Gauss function or hyperfine line, which is introduced into the calculation for the spectral simulation, is expressed as "intrinsic line width."

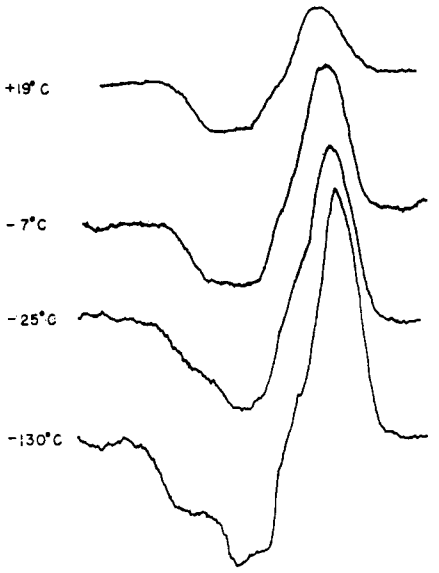

FIGURE 3: Temperature dependency of electron paramagnetic resonance spectrum of Hb α-NO. Experimental conditions as for Figure 2.

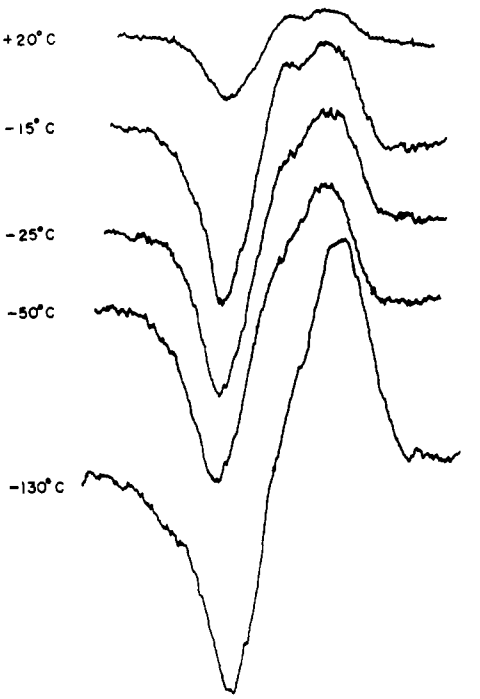

FIGURE 4: Temperature dependency of electron paramagnetic resonance spectrum of Hb β-NO. Experimental conditions as for Figure 2.

and Hb β-NO are highly asymmetric and their apparent g values and the width between maximal deflection points are different.

The principal g values of Hb α-NO and Hb β-NO estimated from the simulation are listed in Table I. Evidently the electron paramagnetic resonance signal of Hb α-NO shows axial symmetry, whereas that of Hb β-NO deviates slightly from the axial symmetry. In the spectral simulation, the line width of the best-fit component Gauss function was 40 gauss for both the subunits. Alternatively, when nitrogen anisotropic hyperfine couplings were introduced into the calculation, their component line width could not be reduced below 30 gauss for any combination of anisotropic hyperfine coupling constants.

Low-Temperature Spectra. As reported previously (Shiga *et al.*, 1968), remarkable differences in electron paramagnetic resonance spectra were observed between Hb α-NO and Hb β-NO at low temperatures below $-50°$ as well. Again, the asymmetric electron paramagnetic resonance signal of Hb A-NO and erythrocyte NO was simply the sum of the signals of the isolated chain NO's. Hb β-NO showed a nearly symmetric signal having three different principal g values. In contrast, the signal of Hb α-NO was highly asymmetric, also with three different principal g values. Their g values are listed in Table I.

TABLE I: Principal g Values of Electron Paramagnetic Resonance Spectra of Hb α-NO and Hb β-NO.

	Room Temp (above 0°)	Low Temp (below −50°)
Hb α-NO	2.000, 2.000, 2.045	1.987, 2.017, 2.074
Hb β-NO	2.001, 2.004, 2.045	2.000, 2.017, 2.045

Temperature Dependency of the Spectra. Change of the temperature markedly altered the shape of electron paramagnetic resonance signals of all the Hb-NO's. On lowering the temperature, the signal of Hb α-NO changed dramatically from an axially symmetric form to a highly asymmetric one, as shown in Figure 3. At around $-20°$, the axially symmetric signal began to change and the change to a highly asymmetric signal was completed at $-50°$. Similarly, the signal of Hb β-NO changed from the slightly asymmetric form to the nearly symmetric form at around $-20°$, but with less sharp transition (Figure 4). The electron paramagnetic resonance signals of Hb A-NO and erythrocytes NO also changed with temperature, holding the additivity of the constituent subunits. In Figure 5, the shifts of g values with temperature are illustrated. A linear relation was observed between the electron paramagnetic resonance absorption (area under the integrated spectrum) and $1/T°K$.

The Effects of Urea and pH. As seen in Figure 6, the addition of 4 M urea significantly modified the signal shape of Hb β-NO at room temperatures; the signal became more axially symmetric and/or its component line width increased. The change began within 5 min after the addition and gradually proceeded for about 2 hr. On the other hand, the signal of Hb α-NO showed no detectable change within 90 min after the addition of urea at room temperatures, but it became very broad 3 hr after the addition.

Above pH 10, the signal of Hb β-NO immediately became rather axially symmetric and broad at room temperatures, whereas the signal shape of Hb α-NO remained

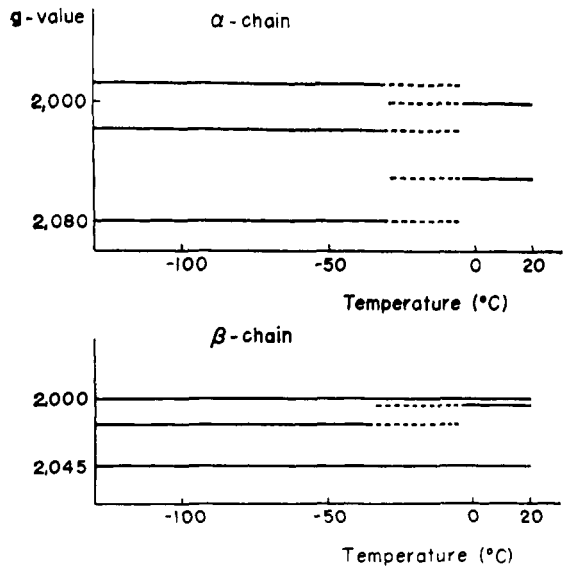

FIGURE 5: Shifts of principal g values with temperature.

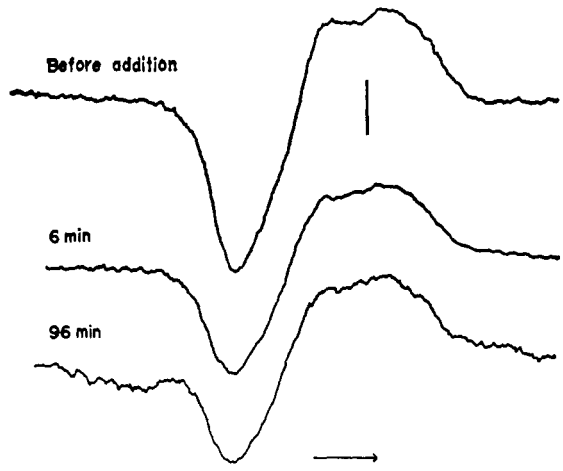

FIGURE 6: Effect of 4 M urea on electron paramagnetic resonance spectrum of Hb β-NO at 19°. Modulation amplitude, 4 gauss; time constant, 0.3 sec. Figures over the curves indicate the time after addition of urea.

unchanged. The effect of acidic pH (below 4.5) could not be studied, because of a considerable denaturation and precipitation of the proteins.

These results indicate that the modification of the protein conformation alters the electron paramagnetic resonance signals of Hb-NO's to a more axially symmetric and/or more broad form.

Saturation Behavior against Microwave Powers. At any temperatures, the electron paramagnetic resonance signals of all the Hb-NO's never saturate with increasing applied microwave power up to 200 MW at the entrance of the H_{012} cavity. The line shapes also remain unchanged with the increase of the microwave power.

Discussion

The magnetic susceptibility of Hb A-NO was determined as 1.75 BM by Coryell *et al.* (1939). The electron paramagnetic resonance signal of Hb A-NO was first described by Bennett *et al.* (1955) and has been confirmed by other investigators (Ingram and Bennett, 1955; Sancier *et al.*, 1962; Rein *et al.*, 1964; Shiga *et al.*, 1968). It can be safely argued that the detected paramagnetism originates from NO molecules bound to heme, since the binding capacity of Hb A for NO and CO is exactly the same (Hermann, 1865; Gibson and Roughton, 1957b) and NO dissolved in the buffer solution used does not give any electron paramagnetic resonance signal.

The present observation on Hb A-NO agreed well with the previously reported results, *i.e.*, a singlet electron paramagnetic resonance signal at g = 2.00–2.03 with a broad apparent line width. We found a complicated structure on the second derivative of the low-temperature spectrum, which differed from the distinct three-line spectrum demonstrated by Gordy and Rexford (1961) and assumed as the nitrogen hyperfine couplings by them. Since the spectrum of Hb A-NO (either native or reconstituted) can be composed by the simple addition of the signals of the two subunit NO's, a possible appearance of such structures due to overlapping of the two types of spectrum must be considered. The fact that the spectrum of Hb A-NO can be composed by a simple addition of Hb α-NO and Hb β-NO suggests that the electronic structure and orientation of NO are not altered by subunit interaction. However, present data obtained on hemoglobin solutions by the X-band apparatus would not be sufficient to draw a decisive conclusion and further accurate measurements on hemoglobin crystals with a Q-band apparatus seem to be needed.

Griffith (1956) has made a theoretical calculation of principal g values of Hb A-NO based on a model in which the NO molecule situates its NO axis parallel to the heme plane. According to his model, the electron paramagnetic resonance signal should show axial symmetry, *i.e.*, two out of three principal g values coincide. A similar view was also given by Lang and Marshall (1966). Substitution of the g values for Hb α-NO at room temperature into the Griffith equation for the energy difference between the two antibonding π orbitals of NO gives the figure of about 1.8 eV, which favors the "parallel to heme" structures.

As mentioned in the Experimental Section, some uncertainty remained in the determination of principal g values by the simulation. The best-fit spectrum based on the proposed best set of g values was obtained only with a rather broad Gauss function (intrinsic line width, 40 gauss). Introduction of nitrogen hyperfine coupling constants greatly increased the discrepancy between the observed and simulated spectra. Moreover, when the intrinsic line width of the hyperfine couplings was reduced below 5 gauss, the simulated spectrum exhibited three sharp lines (Figure 1h). This type of spectrum has been observed in the microsome–NO complex (H. S. Mason, 1967, personal communication), but never found in our present study on Hb-NO's. From these reasons an attempt to estimate the coupling constants was abandoned. A broad intrinsic line width does

not directly mean a broad component line width, since unresolved hyperfine couplings due to nitrogens of NO, heme, and histidine, and/or uncertainty in g values also inevitably require the introduction of Gauss function with a broad intrinsic line width.

The uncertainties in the determination of g and A values and the broadness of the best-fit intrinsic line width can be explained by several ways. (1) Lunsford (1967) explained the broadening of electron paramagnetic resonance signals of NO adsorbed on MgO by assuming that the NO molecule is in a dissociation–association equilibrium state. This possibility, however, is very unlikely, since the dissociation velocity of NO from hemoglobins has been shown to be very slow, the half-time of the dissociation being *ca.* several hours (Gibson and Roughton, 1957b). Further, the above-mentioned saturation behavior of the electron paramagnetic resonance signals of Hb-NO's against microwave power also suggests that the NO molecule is strongly bound on the iron atom of heme, having a short relaxation time. (2) Eleven amino acids make a van der Waals contact with heme in hemoglobin (Perutz, 1965) and some more amino acids may contact with liganded NO molecules. Therefore, the thermal movement of these amino acids, as well as that of heme itself, leads to a randomness in the orientation of the NO molecule, even if it is tightly fixed to heme. As pointed out by Lefevre and Maruani (1965), these effects yield the broadening of intrinsic line widths. (3) A slow molecular tumbling of hemoglobin molecule as a whole also will cause the line broadening. However, the fact that the line broadening was observed even in a frozen state does not support the view. (4) Relaxation time broadening can be ruled out, since the intrinsic line width appears to be independent of temperature. Thus, it can be reasonably assumed that the orientation of NO molecules to the heme plane is not uniquely fixed but has some randomness, giving an approximate principal g values and also an ambiguity of A values. Supporting this view, Eisenberger and Pershan (1967) have proposed that a "misorientation" of heme in ferrimyoglobin crystals is sufficient to explain the observed angular dependence of the line width.

As indicated in Figure 5, the estimated g values for Hb α-NO and Hb β-NO changed with temperature discontinuously. This is not due to the change in spin state of iron, since the areas under integrated electron paramagnetic resonance signals increased linearly with $1/T$ in the experimental temperature range. Therefore, the change of the g values can be considered to reflect an altered orientation of the NO molecule at room and low temperatures. At room temperatures, the orientation in Hb α-NO may be best explained by Griffith's "parallel to heme" structure as mentioned already, whereas NO attached to Hb β deviates slightly from the parallel structure. The distinction may arise from the difference in the steric restriction around liganded NO in both subunits, which may be related to the difference in the protein conformation. At low temperatures, NO combined with both subunits seems to deviate from the parallel structure considerably and the deviation is more marked in Hb α-NO than in Hb β-NO. Sharp transitions observed at -20 to $-50°$ suggest that only two types of orientation of NO, *i.e.*, the room and low temperature types, are allowed for the subunit NO's and that freezing of some essential structures determining the orientation induces a more distorted orientation and further decrease of temperatures does not affect the orientation. In this connection, it is worthwhile mentioning the results of Mössbauer experiments performed by Lang and Marshall (1966), which revealed that NO the molecule in mouse Hb-NO situates at a fixed acute angle to the heme plane between 1.2 and 190°K.

On the other hand, urea and high pH seem to remove the steric or librational restriction on the NO molecule in Hb β-NO, giving a signal of nearly axial symmetry and/or broad intrinsic line width.

These results clearly indicate an intimate relation between the conformation of proteins and the orientation of ligands combined to them and also a distinct difference in the orientation at room and low temperatures.

Acknowledgments

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Biochemistry of the Sphingolipids. XVIII. Complete Structure of Tetrasaccharide Phytoglycolipid*

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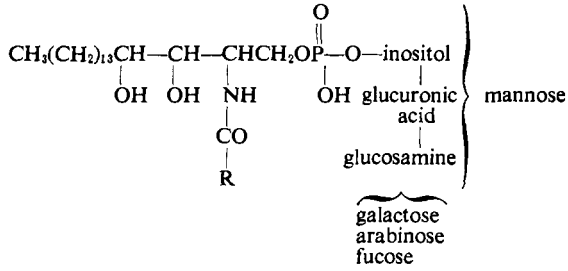
ABSTRACT: The tetrasaccharide (2-mannosido-6-[D-glucosamido-(1–4)-D-glucuronido]inositol) obtained from the hydrolysis of phytoglycolipid has been oxidized with periodate and the products reduced with sodium borohydride. Isolation of D-arabitol as one of the polyol products shows that in the tetrasaccharide the inositol is 2,6-disubstituted. Proton magnetic resonance studies on the derived glucosylinositol and carboxyl-reduced *N*-acetyl trisaccharide showed that the glucuronic acid moiety was attached to the 6 position of inositol. The mannose, therefore, must be attached to the 2 position of inositol. Also deduced from proton magnetic resonance spectra was the all-α configuration of the tetrasaccharide.

Confirmation of this came from the time of half-hydrolysis of the *N*-acetyl trisaccharide. The point of attachment of the phosphate to the inositol in phytoglycolipid was shown to be through the 1 position by oxidation studies on the intact phytoglycolipid. The latter point was substantiated by mild acid hydrolysis of phosphorylated oligosaccharide to afford only inositol 1-phosphate.

Previous studies from these laboratories (Carter *et al.*, 1958a,b, 1964a,b) have established the following type structure for phytoglycolipid, a complex glycolipid widely distributed in plant seeds.



I, R = fatty acid residue

Alkaline hydrolysis of phytoglycolipid from corn gave a mixture of oligosaccharides which could be separated on Dowex-2 (HCO_3^-) ion-exchange resin into a series of fractions eluted in order of decreasing molecular weight. The last was the trisaccharide, glucosamido-glucuronidoinositol. This nicely crystalline substance is readily obtained in excellent yield, by acid hydrolysis, from all the higher oligosaccharides and accounts for most if not all of the glucosamine and glucuronic acid present in these materials. The major peak (40% of the total) was a tetrasaccharide containing mannose as the fourth component. This amorphous tetrasaccharide was characterized as the crystalline *N*-acetyl- and *N*-acetylcarboxyl-reduced derivatives. Penta-, hexa-, hepta-, and octasaccharides were also obtained in small amounts and partially characterized by analysis and paper chromatography. These higher oligosaccharides contain galactose, arabinose, and fucose (in the case of flax) in varying amounts in addition to the mannose, glucuronic acid, glucosamine, and inositol of the tetrasaccharide.

In this paper are reported studies which have established the complete structure of the tetrasaccharide (and thereby that of the trisaccharide) and also that of the parent phytoglycolipid.

During the course of attempts to determine the structure of the oligosaccharides numerous periodate oxidation studies have been made on the oligosaccharide mixture and purified tetrasaccharide. The results were not conclusive and their interpretation was complicated by extensive overoxidation occurring under a variety of

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