The Nature, Quantity, and Mode of Attachment of Hexoses in Ichthyocol*

OLGA O. BLUMENFELD,† MERCEDES A. PAZ,‡ PAUL M. GALLOP, AND SAM SEIFTER

From the Department of Biochemistry and the Unit for Research in Aging, Albert Einstein College of Medicine, Yeshiva University, New York 61, New York

(Received for publication, June 24, 1963)

Small amounts of components which react as hexoses are present in highly purified collagens. Grassmann et al. (1) have shown that the hexose units are probably attached to the protein backbone in collagen through a glycosidic linkage. From acid hydrolysates of collagens of a number of species, Gross, Dumsha, and Glazer (2) isolated n-glucose and n-galactose. Certain of the collagens also contained traces of other hexoses, pentoses, and amino sugars. These authors showed that the total carbohydrate content of collagens varied with species, being 0.6 to 2% of molecular weight 320,000. Evidence is also presented which suggests that the hexoses are bound through their C-1 positions in covalent linkages to the protein.

In this communication evidence is presented that ichthyocol contains 1.4 ± 0.1 residues of glucose and 2.1 ± 0.3 residues of galactose per 1000 amino acid residues, corresponding to 5 to 15.5% in vertebrate, and 5 to 15.5% in invertebrate, collagens. Since hexoses have been considered as possible participants in ester cross-linkages of collagen (3, 4), precise information is required concerning their amounts and mode of linkage in the protein.

EXPERIMENTAL PROCEDURE

Materials—Ichthyocol was prepared from the tunics of carp swim bladders as described by Gallop (5). Gelatin solutions were obtained by heating for 10 minutes at 60° and centrifugation in a Spincos model L centrifuge at 100,000 × g at 40° for 20 minutes. The concentration of protein in solution was computed from Kjeldahl nitrogen determinations.

Anthrone, n-mannose, and β-cellulose were purchased from Matheson, Coleman, and Bell, 3-N-methyl-2-benzothiazolone hydrazide hydrochloride from the Aldrich Chemical Company, α-galactose from Merck and Company, α-lactose from Mallinckrodt Chemical Works, and α-glucose and α-D-methylglucoside from Mann Research Laboratories. Purified glucose oxidase (type II) was purchased from Sigma Chemical Company. Galactose oxidase was a generous gift from Drs. John Sandson and Lester Bernstein. Other chemicals used were reagent grade.

Anthrone Reaction—Hexose determinations were carried out on 5- to 10-ng samples of ichthyocol by the method of Seifter et al. (6) with the anthrone reagent.

Separation of Neutral Sugars from Ichthyocol—Ichthyocol (300 mg) was gelatinized and hydrolyzed by heating with 9 ml of 2 N HCl at 110° for 135 minutes in vials sealed under reduced pressure. The excess HCl was removed by evaporation under reduced pressure in a rotary evaporator, and the residue was dissolved in a small volume of water. Neutral sugars were separated from peptides and amino acids on a Dowex 50-X2 column (200 to 400 mesh) in the H+ form, with water as eluent. The anthrone-reacting hexose material was not retained on resin, and emerged free of ninhydrin-reacting material. Recovery of anthrone-reacting material averaged about 80%.

Paper Chromatography of Sugars in Isolated Carbohydrate Portion Sugars were chromatographed on Whatman No. 1 paper in 1-butanol-pyridine-water (6:4:3), butanol-acetic acid-water (4:1:5), and butanol-ethanol-water (10:1:2). Descending chromatograms were prepared, the solvent being allowed to run off the paper. Hexose spots were made visible by means of an aniline-phthalate spray.

Specific Colorimetric Procedures—Hexosamines were determined by a modified Elson-Morgan method (7) and by chromatography on Amberlite IR-120 columns after hydrolysis of the protein for 22 hours in 6 N HCl at 105° in tubes sealed under reduced pressure. Methyl pentoses were assayed by the cysteine-sulfuric acid method (8), hexuronic acids by the carbazole method (9), and formaldehyde by the chromotropic acid method (10). Sialic acid was determined by the resorcinol method as described by Svennerholm (11, 12), by direct Ehrlich reaction (13), and by the thiobarbituric acid reaction after periodate oxidation (14). The thiobarbituric acid (14) and diphenylamine (15) tests were used for determination of 2-deoxy sugars.

Quantitative Estimation of Glucose in Isolated Carbohydrate Fraction—The neutral sugar eluate from the Dowex 50 column was analyzed for glucose by use of glucose oxidase. The assay was carried out in 0.16 m acetate buffer, pH 5.1, at 37° with 0.4 to 0.7 mg of enzyme in a total reaction volume of 6 ml. Glucose (2.0 μmoles) was used as standard; galactose and n-mannose were used as controls and were unaffected by the enzyme. Approximately 2 μmoles of glucose color equivalents of anthrone-reacting material in the isolated sample were used for the assay. An aliquot of this magnitude is derived from about 74 mg of ichthyocol. At different time intervals an anthrone determination was carried out on 1 ml of the enzyme reaction mixture; an enzyme blank contained a small amount of anthrone-reacting enzyme.
Galactose and mannose give, respectively, 54.4% and 52.0% of the color given by glucose in the anthrone reaction.

Quantitative Estimation of Galactose in Isolated Carbohydrate Fraction and in Intact Ichthyocol—α-Galactose oxidase of Polyporus circinatus (16) was used. The assay was carried out in 0.03 M Tris buffer, pH 7.0, at 37°C. At definite time intervals the amount of aldehyde produced by the action of the enzyme through oxidation of position 6 of galactose was measured by the aldehyde method of Sawicki et al. (17), modified as described below.

To 0.2 ml of sample (containing about 0.06 μmole of aldehyde), 0.4 ml of water was added and the pH was adjusted to about 4.0. The volume was brought to 0.8 ml with water, and 0.2 ml of 1% aqueous N-methylbenzothiazolone hydrazine hydrochloride was added. The mixture was heated for 3 minutes at 100°C and cooled to room temperature; 2.5 ml of 0.2% FeCl3 were then added. After 5 minutes the volume was adjusted to 10 ml with acetone and the optical density was read at 670 μM against a reagent blank in a Coleman junior spectrophotometer.

Estimation of Products of Periodate Oxidation of Ichthyocol—A 2.5% solution of ichthyocol (2 ml) adjusted to pH 3.5 was treated with 0.02 M periodic acid at pH 3.5 in the dark at room temperature. The excess periodate was removed by stirring with Dowex 1-X8 (acetate form, 20 to 50 mesh). α-Lactose, β-cellobiose, and α-α-methylglucoside served as controls and were treated similarly with periodate.

For a qualitative examination of products after removal of periodate, 1 ml of the reaction mixture was heated at 100°C for 5 minutes with 1 ml of a 0.5% solution of 2,4-dinitrophenylhydrazine in 4 N HCl. Standard solutions of α,β-glyceraldehyde, glyoxal, α-d-erythrose, and formaldehyde were treated in an identical manner. Precipitates were obtained which consisted mainly of 2,4-dinitrophenylhydrazones; these were collected by centrifugation, washed twice with 2 N HCl, dissolved in redistilled ethyl acetate, and chromatographed on thin layers of silica gel.

Toluene-glacial acetic acid-water (4:3:1) and methylecyclohexane-ethyl acetate-acetonitrile-water (65:35:10:1) were used as developing solvents. The chromatograms were observed with ultraviolet light and then treated with alcoholic 1 N NaOH.

A semiquantitative spectrophotometric estimation of glyoxal was made after conversion to an “osazine” of glyoxal with N-methylbenzothiazolone hydrazine (Scheme 1). To 1.0 ml of sample, containing from 0.01 to 0.05 μmole of glyoxal, were added 4.0 ml of 50% glacial acetic acid and 0.2 ml of 1% N-methylbenzothiazolone hydrazine. The solutions were heated for 15 to 40 minutes at 80°C and read at 400 μM in a Coleman junior spectrophotometer. It was found that glyoxal was liberated slowly from its glycosidic attachment, and in some instances longer heating was required. Azines obtained by reaction of N-methylbenzothiazolone hydrazine hydrochloride with aldehydes have negligible absorption at 400 μM, as seen in Fig. 1, in which are presented spectra of known compounds in 50% acetic acid-water.

Aldehydes produced on periodate oxidation were measured by the modified colorimetric Sawicki method described above (as employed in the galactose oxidase assay). Amino acid analyses were carried out by the automatic technique of Spackman, Stein, and Moore (18) after hydrolysis of the protein under reduced pressure in 6 N HCl at 105°C for 22 hours.

RESULTS

Nature and Quantity of Hexoses in Ichthyocol—The results of the anthrone determination on several ichthyocol preparations indicate that 2.5 ± 0.2 residues of glucose equivalents are present per 1000 amino acid residues.1 This value is in agreement with that found by Gross et al. (2) in carp swim bladder collagen.

When the isolated neutral sugar fraction was chromatographed on paper, only two spots were observed, which corresponded to glucose and galactose. The relative concentrations of the two hexoses were obtained from the assays with the specific oxidases. Glucose oxidase oxidizes glucose at position 1, giving rise to gluconic acid, and is without effect on galactose. The anthrone reaction depends on the presence of an aldehyde function at

1 The average weight of one residue in collagen is 92 mg.
position 1 of an aldopyranose, and is negative with glucosonic acid. The action of glucose oxidase on the neutral sugar fraction of ichthyocol decreased the anthrone-reacting material to a value corresponding to the galactose present. A typical experiment is shown in Fig. 2. It can be seen that the color intensity obtained with anthrone, after glucose oxidase action, decreased and finally leveled off at 46% of the initial value. The amounts of galactose and glucose, and their ratio, can be calculated from this value and from the color yields of glucose and galactose in the anthrone reaction. These results are included in Table I.

Similar results were obtained when the isolated carbohydrate fraction was assayed with galactose oxidase. This enzyme catalyzes oxidation at position 6 of galactose (16), giving rise to a new free aldehyde group which can be measured with the Sawicki procedure. Aldopyranoses, such as glucose or galactose, do not react as aldehydes in the Sawicki method. The results of this experiment are shown in Fig. 3. It can be seen that the amount of reactive aldehyde finally formed was equal to the amount of galactose present initially in the sample. The color yield of the new aldehyde produced was determined from a known galactose standard treated with galactose oxidase in an identical manner, and was approximately 60% that given by acetaldehyde on reaction with N-methylbenzothiazolone hydrazone hydrochloride. The quantities of galactose and glucose and their molar ratios, calculated from these experiments, are included in Table I.

Because of the good agreement between results obtained with the two oxidases, one may conclude that glucose and galactose, but no other anthrone-reacting components, are present in ichthyocol. Free and acetylated hexosamines, fucose, 2-deoxy sugars, hexuronic acids, and sialic acid could not be detected by application of specific colorimetric tests to the protein or its various hydrolyzates. Also, hexosamines could not be detected in column chromatograms obtained with the amino acid analyzer.

**Table I**

| Contents of glucose and galactose in ichthyocol determined by actions of specific oxidases on hexose fraction of that protein |
|---|---|---|
| **Residues of hexose per 1000 residues of amino acids** | **Ratio of galactose to glucose** |
| Galactose | Glucose |
| Using glucose oxidase (Experiment I) | 2.06 | 1.37 | 1.50 |
| Using glucose oxidase (Experiment II) | 2.11 | 1.34 | 1.57 |
| Using galactose oxidase | 2.00 | 1.55 | 1.48 |
| Average of above | 2.06 | 1.35 | 1.52 |

* A component of unknown structure reacting as an aldehyde is present in ichthyocol and other collagens and can be detected after treatment of the protein with nucleophilic reagents which cleave the easier attachments (19).
Hexoses in Ichthyocol

Vol. 238, No. 12

PERIODATE TREATED

Fig. 4. Chromatography of 2,4-dinitrophenylhydrazine derivatives of standards and products obtained on periodate oxidation of lactose, cellobiose, \( \alpha\)-\( \beta\)-methylglucoside, and ichthyocol; toluene-glacial acetic acid-water (4:3:1); thin layer of silica gel.

TABLE II

Products obtained after periodate oxidation of ichthyocol, lactose, cellobiose, and \( \alpha\)-\( \beta\)-methylglucoside

Conditions of oxidation were: 0.02 M periodate, pH 3.5, 4 hours in the dark, room temperature. For ichthyocol, the results are expressed as moles per 1000 residue moles of amino acid. For lactose, cellobiose, and \( \alpha\)-\( \beta\)-methylglucoside, the values given are moles produced per mole of compound oxidized.

<table>
<thead>
<tr>
<th>Products produced at</th>
<th>Glycol</th>
<th>Formaldehyde</th>
<th>Total aldehyde</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ichthyocol</td>
<td>2.22</td>
<td>7.5</td>
<td>13.2</td>
</tr>
<tr>
<td>Lactose</td>
<td>0.78</td>
<td>0.0</td>
<td>2.36</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>0.69</td>
<td>0.08</td>
<td>2.06</td>
</tr>
<tr>
<td>( \alpha)-( \beta)-Methylglucoside</td>
<td>0.62</td>
<td>0.11</td>
<td>1.32</td>
</tr>
</tbody>
</table>

* Liberated by heating at 80° in 50% acetic acid for 40 minutes.
† For ichthyocol, total aldehyde produced includes aldehydes arising both from hexoses and from hydroxyxyl residues (formaldehyde and \( \alpha\)-aminoglutaric semialdehyde); the value is calculated with acetaldehyde as a standard. For lactose, cellobiose, and the \( \alpha\)-\( \beta\)-methylglucoside, the identity of products was established by thin layer chromatography; all products were considered to occur in equimolar amounts, and total aldehyde was then calculated by means of previously established molar color yields. Thus, the relative molar color equivalents, in the Sawicki reaction, are, respectively, 1.0, 0.47, and 0.28 for glyceraldehyde, erythrose, and glyoxal.

†† If one relates the amount of glyoxylic produced to the amount of hexose in ichthyocol (3.5 residues of hexose per 1000 amino acid residues; see Table I), the value becomes 0.63 mole of glyoxylic per mole of total hexose.

Fig. 5. Action of galactose oxidase on intact ichthyocol (○) and on a galactose standard (●). Incubation was in 0.03 M Tris buffer, pH 7.0, at 37°. Aldehyde produced was measured with the Sawicki reagent and referred to an acetaldehyde standard.

of lactose or cellobiose and about 70% of 1 equivalent of glucose and glyoxal per mole of \( \alpha\)-\( \beta\)-methylglucoside were released. The 2,4-dinitrophenylhydrazones of these products were identified by thin layer chromatography (see Fig. 4). The value obtained for total aldehyde after periodate oxidation of ichthyocol includes formaldehyde and probably \( \alpha\)-aminoglutaric semialdehyde residues; these products arise via the oxidation of hydroxyxyl residues. Absence of hydroxyxylsine in acid hydrolysates of periodate-treated ichthyocol was demonstrated by amino acid analysis.

Since the amount of glyoxylic released from periodate-oxidized ichthyocol was almost equal to that released from appropriate model compounds, it can be concluded that both hexoses in ichthyocol underwent oxidation at their carbon atoms 2 and 3.

To determine whether the hydroxyl group of position 6 of galactose present in ichthyocol is free, gelatin derived from ichthyocol was treated directly with galactose oxidase as described above. The course of this oxidation with enzyme is shown in Fig. 5. Although the reaction on the intact protein was slower than with the isolated hexose fraction, 82% of the previously determined amount of galactose was oxidized in 24 hours. The hydroxyl at position 6 of galactose is available to the enzyme, and therefore unsubstituted.

DISCUSSION

Ichthyocol contains 1.4 ± 0.1 residues of glucose and 2.1 ± 0.2 units of galactose per 1000 amino acid residues, corresponding, respectively, to 5 residues of glucose and 7 of galactose per tropocollagen molecule (mol. wt. 320,000). Experiments with glucose and galactose oxidases show that the total hexoses of ichthyocol, as determined with anthrone, can be accounted for as glucose and galactose. Hexosamines, deoxy sugars, sialic acids, fucose, or other sugars which do not react with anthrone are not present in ichthyocol, as indicated by specific colorimetric tests. This latter finding is in agreement with results obtained by others with purified collagens (2, 20).

Since collagen is obtained from tissues which are rich in carbohydrates, the question often is raised whether the carbohydrate present in small amount in collagen preparations is an integral part of the molecule. Grassmann et al. (20) found that the quantity of hexoses measured with the anthrone reaction decreased during successive steps of purification of bovine col-
lagen, but finally attained a constant value in "pure" collagen. Gross et al. (2) also found hexoses in highly purified collagens from diverse sources. Our experiments confirm the integral occurrence of glucose and galactose, and their mode of attachment in the collagen molecule now becomes of great interest.

The occurrence of protein-bound glyoxal in ichthyocol which has been treated with periodate permits the inference that the hexoses are attached to the protein through their C-1 atoms. The release of glyoxal from this attachment occurs by acid hydrolysis in the course of the colorimetric determination with N-methylbenzothiazolone hydrazone hydrochloride, and the rate of this release from periodate-oxidized ichthyocol is almost identical with that from cellobiose, lactose, or α-D-methylglucoside treated with periodate. Thus the hexoses would appear to be bound in ichthyocol through glycosidic linkages. Although identical with that from cellobiose, lactose, or α-D-methylglucoside, is also inferred from the products obtained after oxidation with periodate.

The occurrence of protein-bound glyoxal in ichthyocol which has been treated with periodate permits the inference that the hexoses are attached to the protein through their C-1 atoms. The release of glyoxal from this attachment occurs by acid hydrolysis in the course of the colorimetric determination with N-methylbenzothiazolone hydrazone hydrochloride, and the rate of this release from periodate-oxidized ichthyocol is almost identical with that from cellobiose, lactose, or α-D-methylglucoside treated with periodate. Thus the hexoses would appear to be bound in ichthyocol through glycosidic linkages. Although identical with that from cellobiose, lactose, or α-D-methylglucoside, is also inferred from the products obtained after oxidation with periodate.

The fact that glyceraldehyde, but not erythrose, was also obtained after periodate treatment of ichthyocol suggests that the oxidation involved hydroxyl groups on positions 2 and 3, and 3 and 4, of the hexoses. Although isolation of products was not quantitative, the exclusive occurrence of glyceraldehyde and glyoxal indicated that the hydroxyl groups on positions 2, 3, and 4 of either glucose or galactose components of ichthyocol are unsubstituted. In the case of galactose, information that the hydroxyl group on position 6 is also unsubstituted, revealed by the use of galactose oxidase, compels the conclusion that the hexose is bound to the protein through position 1. Because it is probable that the hexose occurs as a pyranose structure, a further inference is that galactose does not supply hydroxy groups for cross-linkages in ichthyocol.

Collagen contains about six ester bonds (22), 2.1 galactose units, and 1.4 glucose units per 1000 amino acid residues. Participation of the galactose moieties in ester linkages has already been eliminated. Occurrence of the glucose units in aldopyranose structures would leave only position 6 of this hexose unaccounted for and, therefore, the sole position which could participate in the ester groups. Even if glucose were involved in this manner, it could only account for one to two of the total of six ester linkages per 1000 amino acid residues.

Thus, the evidence suggests that both glucose and galactose are bound exclusively via a glycosidic linkage to ichthyocol. That the two hexoses occur as monosaccharides and not associated in disaccharide or polysaccharide units, is also inferred from the products obtained after oxidation with periodate.

**SUMMARY**

1. Ichthyocol contains 1.4 ± 0.1 residues of glucose and 2.1 ± 0.2 residues of galactose per 1000 amino acid residues, corresponding to 5 residues of glucose and 7 of galactose per tropoel lagen molecule of molecular weight 320,000. These results were obtained with the use of glucose and galactose oxidases on a neutral carbohydrate fraction isolated from ichthyocol hydrol ysatcs.

2. A new highly sensitive method for assaying galactose oxidase has been developed. The aldehyde produced is measured by the procedure of Sawicki, Hanauer, Stanley, and Elbert (17).

3. Specific colorimetric tests indicate that hexosamine, deoxy sugars, sialic acids, fucose, and carbohydrates, other than glucose and galactose, which react with anthrone are not present in ichthyocol.

4. From identification of products obtained after periodate oxidation it can be concluded that glucose and galactose are attached to the protein through a glycosidic bond, and that the hydroxyl groups at positions 2, 3, and 4 of both hexoses are unsubstituted. The hydroxyl at position 6 of galactose is also unsubstituted, as shown by assay with galactose oxidase on the intact protein.

5. From these results, it appears that glucose and galactose are present in ichthyocol as monosaccharides and probably do not participate in covalent cross-linking.

**REFERENCES**

The Nature, Quantity, and Mode of Attachment of Hexoses in Ichthyocol
Olga O. Blumenfeld, Mercedes A. Paz, Paul M. Gallop and Sam Seifter


Access the most updated version of this article at http://www.jbc.org/content/238/12/3835.citation

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/238/12/3835.citation.full.html#ref-list-1