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THE DISTRIBUTION, NATURE AND LINKAGE OF SIALIC ACIDS IN SKIN PROTEINS

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SUMMARY

1. The total content of sialic acids of the individual protein constituents of skins and hides, *viz.*, collagen, procollagens, elastin, albumin, globulin and mucoid were quantitatively estimated by resorcinol and thiobarbituric acid methods. Of these proteins, the sialic acid content of mucoid was found to be the highest and that of citrate-soluble procollagen the lowest.

2. The component sialic acids of each protein were identified and quantitatively estimated by a paper-chromatographic method which was standardised. *N*-Acetylneuraminic acid in higher concentration and *N*-glycolylneuraminic acid in lower concentration were found to occur in skin albumin, globulin or mucoid whereas only *N*-acetylneuraminic acid could be detected in collagen, procollagens and elastin.

3. The sialic acids were found to be easily released from these proteins by the action of purified neuraminidase (*N*-acetylneuraminate glycohydrolase, EC 3.2.1.18) of *Clostridium perfringens*, which indicated that the sialic acids were bound terminally in *O*-glycosidic linkages in these proteins.

4. The major glycosidic partner for sialic acids in collagen, albumin, globulin or mucoid was identified by estimating the component hexoses and hexosamines of the original protein as well as the sialic acid-free protein before and after periodate oxidation. It was found that the reducing group of sialic acid was joined in *O*-glycosidic linkages to the galactose residues in these proteins.

INTRODUCTION

In natural products sialic acids are usually found together with hexoses and hexosamines. The protein constituents of skins and hides are known[1] to contain different proportions of hexoses and hexosamines. The ground substance of animal skin was reported[2] to contain polysaccharide–protein complexes, the polysaccharide moiety of which was composed of different hexoses, hexosamine and sialic acid. The sialic acid content of most of the collagen samples was found[3] to be less than 0.1 %, except in calf skin (0.2 %), swim-bladder tunic (0.15 %) and swim-bladder elastin (0.3 %). DEASY[4,5] presented evidence for the occurrence of sialic acid or a compound of the sialic acid group in different collagen preparations, the solutions obtained in the puri-

Abbreviations: NANA, *N*-acetylneuraminic acid; NGNA, *N*-glycolylneuraminic acid.

fication of the corium collagen by extraction with several reagents or the insoluble residue left after autoclaving whole hide. WOOD[6] reported the presence of sialic acid in gelatin. DEASY[7] identified pyrrole-2-carboxylic acid in chromatograms of hydrolysates of steer-hide corium-collagen preparations and reported that the compound was probably formed by decomposition of a sialic acid of the hide during acid hydrolysis. Very recently, she estimated[8] the sialic acid content of corium collagen and whole steer hide and identified the sialic acid present in steer-hide hydrolysate as NANA.

The sialic acid of several sialyl compounds is known to be linked terminally in glycosidic linkages through its reducing group. Galactosamine in bovine submaxillary mucoid[9]; galactose in orosomucoid[10], ox-brain mucolipid[11], human plasma Ba-α_2-glycoprotein[12] and trisaccharide[13] of human milk; and lactose in trisaccharide[14] of cow colostrum or rat mammary glands were shown to be the major partners of sialic acids in glycosidic linkages. No information, however, is available about the distribution, nature and the type of linkage of sialic acids in individual protein constituents of skins and hides. In the present investigation, therefore, the results obtained on such studies on skin proteins are presented.

EXPERIMENTAL

Preparation of proteins

Collagen was prepared from the butt portion of a fresh buffalo hide by the method of BOWES AND KENTEN[15] as modified by JOSEPH AND BOSE[16].

Acid-soluble procollagen was prepared from freshly excised tail tendons of albino rats using 0.1 M acetic acid by adopting the method of KESSLER *et al.*[17].

Neutral-salt-soluble procollagen was prepared from young albino-rat skins by following the method of JACKSON[18] and was purified by the method described by JACKSON AND FESSLER[19].

Citrate-soluble procollagen was prepared and purified by the method of JACKSON[18] from the residue left after extraction of the rat skins with NaCl solution.

Elastin was prepared from cattle *Ligamentum nuchae* by the modified method of PARTRIDGE *et al.*[20].

Albumin, globulin and mucoid were prepared from fresh goat skin by the method of DHAR AND BOSE[21] and purified by adopting the usual method of redissolution and reprecipitation of the protein followed by redissolution and dialysis.

Each of the proteins prepared was dried *in vacuo* over P_2O_5 and finely powdered.

Estimation of sialic acids

For the hydrolysis of the protein and removal of interfering substances from the hydrolysate, the method of SVENNERHOLM[22] as adapted by WOLLMAN AND WARREN[23] was followed with some modifications. The conditions of hydrolysis of the protein are of great importance, because heating for longer periods with higher concentrations of acid may cause destruction of sialic acid. In the present study, the sialic acids were liberated by mild hydrolysis of the protein in an evacuated and sealed tube with 0.1 N H_2SO_4 at 80° for 2 h in the case of procollagens, albumin, globulin and mucoid, and at 100° for 4 h in the case of collagen and elastin. In preliminary experiments, it was observed that under the hydrolytic conditions employed, the liberation of sialic acids reached a maximum after about 2 h in the case of

procollagens, albumin, globulin and mucoid and after about 4 h in the case of collagen and elastin. Most of the interfering substances were eliminated by passing the hydrolysate through a column of Dowex-1 in the formate form. The contaminants were washed out with distilled water and the sialic acids which were retained by the column were eluted with 0.6 M formic acid. The eluate was freed of formic acid by lyophilisation and made up to volume with distilled water. The sialic acid content of this solution was determined by following two independent methods, *viz.*, the thiobarbituric acid method of WARREN[24] and the resorcinol method of SVENNERHOLM[22].

Thiobarbituric acid method

An aliquot of the solution was mixed with sodium periodate reagent and kept at 22° for 20 min to oxidise the sialic acids. After treatment with sodium arsenite reagent, the periodate oxidation product was coupled with thiobarbituric acid on heating on a boiling-water bath for 15 min. On cooling, the resulting chromophore was extracted into cyclohexanone. The absorbancy of the clear red cyclohexanone phase was measured in a Beckman DU spectrophotometer at 549 mμ and also at 532 mμ.

Resorcinol method

Another aliquot of the solution was mixed with an equal volume of resorcinol reagent and heated for 15 min on a boiling-water bath. The mixture was cooled, extracted with isoamyl alcohol and the absorbancy of the clear purple alcohol extract was measured in a Beckman DU spectrophotometer at 580 mμ as well as at 450 mμ.

SVENNERHOLM[22] reported that when proteins contain nucleic acids, the acidic eluate obtained from the column-filtration method may also contain ribose and deoxyribose together with the sialic acids. He found that in the resorcinol method, ribose contributes to the absorbancy for sialic acids at 580 mμ and calculated the contribution of ribose from the absorbancy reading at 450 mμ. WARREN[24] observed that in the thiobarbituric acid method deoxyribose which has an absorption maximum at 532 nμ interferes with the measurement of the absorbancy for sialic acids at 549 mμ. Gelatin is known to contain RNA and DNA. For the assay of sialic acids in skin proteins, therefore, absorbancy readings were taken routinely at 580 mμ and 450 mμ in the resorcinol method and at 549 mμ and 532 mμ in the thiobarbituric acid method. The necessary correction for the absorbancy at 549 mμ due to deoxyribose in the thiobarbituric acid assay of sialic acids was made according to the method adopted by WARREN[24]. Similarly, a correction was applied for the absorbancy at 580 mμ due to ribose in the resorcinol method as suggested by SVENNERHOLM[22]. In each method, the amount of sialic acids was calculated as NANA making use of its molar extinction coefficient of 57000 at 549 mμ by the thiobarbituric acid method and 4700 at 580 mμ by the resorcinol method, as reported by WARREN[24]. The results are presented in Table I.

Identification and estimation of component sialic acids by paper chromatography

For the quantitative separation of NGNA from NANA, another aliquot of the solution was subjected to single-dimension paper chromatography (SVENNERHOLM AND SVENNERHOLM[25]) for 20 h on Whatman No. 1 paper previously washed with 0.1 N HCl, distilled water and chloroform–methanol (2:1, v/v) and dried. The solvent

system used was *n*-butanol – *n*-propanol – 0.1 N HCl (1:2:1, v/v). For the development of the chromatogram, the modified thiobarbituric acid spray reagents of WARREN[26,24] were used. After removal of solvent, the paper was sprayed with a solution of 0.05 M sodium periodate in 0.05 N H_2SO_4. After 15 min, a solution consisting of ethylene glycol, acetone and conc. H_2SO_4 (50:50:0.3, v/v) was sprayed on the paper. After 10 min, the paper was sprayed with a solution of 0.6% thiobarbituric acid in 0.5 M Na_2SO_4. It was then heated at 100° for 10 min, when red spots appeared. The sialic acids were identified with reference to the control chromatogram of NANA and NGNA run and developed under identical conditions. Preliminary experiments on paper chromatography of a mixture of NANA, NGNA and deoxyribose by this method showed that deoxyribose did not overlap with the spot of NANA or NGNA.

TABLE I

SIALIC ACID CONTENT OF SKIN PROTEINS

Values expressed as mg NANA/100 g dry protein.

Protein	*Thiobarbituric acid method*	*Resorcinol method*
Collagen	140	143
Acid-soluble procollagen	41	40
Citrate-soluble procollagen	38	38
Neutral-salt-soluble procollagen	0	0
Elastin	66	67
Albumin	518	582
Globulin	870	965
Mucoid	3306	3528

For estimation of the sialic acid, the developed spot was cut out, eluted for 30 min with a mixture of 5 ml H_2O and 5 ml cyclohexanone at room temperature and the absorbancy of the cyclohexanone phase was measured at 549 mμ. The amount of sialic acid in each spot was calculated by reference to a standard absorbancy plot obtained with known amounts of the corresponding sialic acid run and developed under identical conditions. The standard curves for the chromatogram of sialic acids are shown in Fig. 1. The results obtained are presented in Table II.

The method was found to give quite accurate results. The coloured product

TABLE II

QUANTITATIVE PAPER CHROMATOGRAPHY OF SIALIC ACIDS OF SKIN PROTEINS

Protein	*NANA (mg/100 g dry protein)*	*NGNA (mg/100 g dry protein)*	*% total sialic acids*	
			NANA	*NGNA*
Collagen	134	0	100	0
Acid-soluble procollagen	38	0	100	0
Citrate-soluble procollagen	36	0	100	0
Elastin	62	0	100	0
Albumin	380	121	75.9	24.1
Globulin	675	171	79.8	20.2
Mucoid	2909	309	90.4	9.6

formed by reaction with the modified thiobarbituric acid spray reagents could be almost quantitatively eluted with the cyclohexanone–water mixture in about 30 min. WARREN[24] reported that the presence of Na_2SO_4 in the thiobarbituric acid solution facilitated more complete extraction of the sialic acid-chromophore by cyclohexanone.

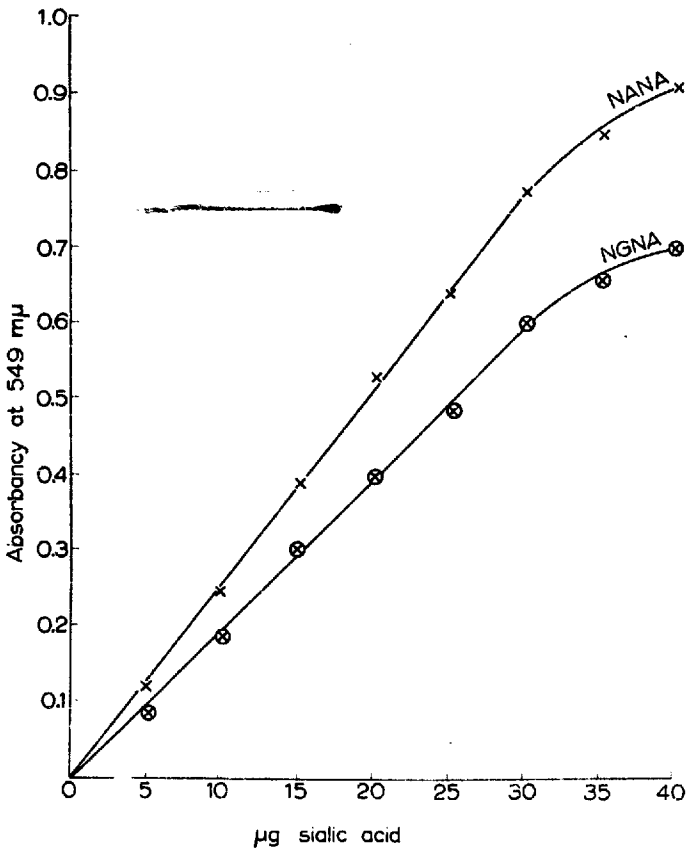

Fig. 1. Standard absorbancy plots for the chromatogram of sialic acids.

Nature of linkage of sialic acids in skin proteins

Since the sialic acids could be easily liberated by mild acid hydrolysis of the skin proteins for the quantitative estimation, it appears that they are bound terminally in acid-labile glycosidic linkages as in many sialoproteins. In order to confirm the nature of linkage of sialic acids in skin proteins, the action of neuraminidase (EC 3.2.1.18), characterised as an *O*-glycosidase and very specific in liberating terminal sialic acids from mucoproteins by the hydrolytic cleavage of *O*-glycosidic links, was investigated. Neuraminidase, isolated from the culture filtrate of *Cl. perfringens* (ATCC strain 10388) and purified by the methods of POPENOE AND DREW[27] and FEENEY *et al.*[28], was used in the present investigation. 25-ml portions of 2% solution or fine suspension of the protein in acetate buffer (pH 4.8) were mixed with 2-ml portions of 0.5% purified neuraminidase solution and incubated at 37° for 0.5 and 4 h at the end of which the mixture was cooled in ice and an equal volume of precooled 5% phosphotungstic acid in 2 N HCl was added. The mixture was kept in the cold for 15 min and the precipitate was removed by centrifugation. The supernatant liquid was analysed for liberated sialic acids by the thiobarbituric acid method

of WARREN[24]. If necessary, the supernatant liquid before analysis was concentrated under vacuum or diluted to a suitable volume according to the concentration of the liberated sialic acids. The results obtained are presented in Table III.

The results show that the sialic acid is attached to the skin proteins through *O*-glycosidic linkages. In order to identify the carbohydrate constituent to which the sialic acid is linked in these proteins, the periodate oxidation method as adapted by POPENOE[10] was followed. POPENOE[29] showed that sodium periodate reacts rapidly with native orosomucoid, apparently primarily with its NANA residues and the action of periodate on the NANA-free mucoid is comparatively slower. When sialic acid residues are cleaved off from proteins by the action of neuraminidase, new groupings susceptible to periodate oxidation are exposed. An analysis of the sialic acid-free protein for the various carbohydrate constituents before and after periodate oxidation would show which one was destroyed by oxidation and thus identify the constituent to which the sialic acid was linked in the original protein.

TABLE III

ENZYMIC LIBERATION OF SIALIC ACIDS FROM SKIN PROTEINS

Protein	*Amount of sialic acids released (mg NANA/100 g dry protein)*		*% sialic acids released**	
	0.5 h	*4 h*	*0.5 h*	*4 h*
Collagen	48	107	34.3	76.4
Acid-soluble procollagen	12	29	29.3	70.7
Citrate-soluble procollagen	11	28	28.9	73.7
Elastin	19	48	28.8	72.7
Albumin	182	408	35.1	78.8
Globulin	290	698	33.3	80.2
Mucoid	889	2506	26.9	75.8

* Calculated with reference to the total sialic acid content of the protein as estimated by the thiobarbituric acid method (Table I).

Hence the proteins, essentially free of sialic acids, were prepared from collagen, albumin, globulin and mucoid by repeated action of the higher concentration of purified neuraminidase and by prolonging the incubation period. The method followed was similar to that adopted by POPENOE AND DREW[27] in their experiments on orosomucoid. The conditions for the reaction of neuraminidase on the proteins were the same as described in the previous experiment except that the reaction mixture was incubated in a dialysis bag and simultaneously dialysed against frequent changes of distilled water; more of neuraminidase solution in acetate buffer (pH 4.8) was added to the reaction mixture after a certain reaction period and the contents of the dialysis bag were mixed frequently. Incubation and dialysis were allowed to proceed until further release of sialic acids could no longer be detected. The sialic acid-free protein was recovered from the non-dialysable material by centrifugation in the case of insoluble protein or by precipitation followed by centrifugation in the case of soluble protein. The proteins were finally dried under vacuum. POPENOE AND DREW[27] carried out ultracentrifugation studies on orosomucoid after enzymic removal of sialic acid residues and reported that there was no gross fragmentation of the protein molecule concomitant with the enzymic reaction. MAYRON *et al.*[30] and RAFELSON *et al.*[31] also observed that no product other than sialic acids was liberated

by neuraminidase action on any of the substrates employed. They could not detect the liberation of free amino acids, sugars or peptides by the neuraminidase action.

The periodate oxidation of the solution or fine suspension of the original protein or sialic acid-free protein in acetate buffer (pH 4.8) was carried out with excess of sodium periodate in the dark at 0° for 5 h. The initial concentration of periodate used was 0.01 M and the protein concentration was 0.5 %. After the reaction, the excess periodate was reduced by ethylene glycol and the mixture was dialysed exhaustively against frequent changes of distilled water. The protein recovered was dried under vacuum. Each protein before and after periodate oxidation was analysed for the constituent hexoses and hexosamines.

Determination of hexose and hexosamine content of proteins and sialic acid-free proteins before and after periodate oxidation

The resin-hydrolysis method was followed for the estimation of the carbohydrate constituents. It is known that sulphonated polystyrene resins such as Dowex-50 act as catalysts for the hydrolysis of proteins.

200 mg of the dried protein were hydrolysed with a suspension of 2.5 g of Dowex-50 in 12 ml 0.1 N HCl in an evacuated and sealed tube at 100° for 24 h. The resin was prepared by repeated washings with 2 N NaOH followed by several washings with 2 N HCl and finally with distilled water. After hydrolysis the suspension was mixed thoroughly and transferred quantitatively to a column of about 10 mm dia., packed at the bottom with glass wool. After collecting the effluent, the column was washed with 15 ml water and the washings were also added to it. After the removal of wash water, the hexosamines were eluted with 20 ml of 2 N HCl followed by 15 ml of distilled water.

The water effluent containing the hexoses was evaporated to dryness *in vacuo* at 40°, the residue was dissolved in a small volume of distilled water and an aliquot was analysed for total hexoses by the modified anthrone method of SCOTT AND MELVIN[32] as adapted by MOSS[33]. The amount of hexoses was calculated as mannose from the standard curve. SCOTT AND MELVIN[32] reported that the anthrone colour is increased in the presence of chloride ions which may be present in the acid hydrolysate. This interference, however, was avoided by evaporation of the effluent *in vacuo*.

The HCl effluent containing the hexosamines was evaporated to dryness *in vacuo* at 40°, the residue was dissolved in 2 ml 0.3 N HCl and analysed for total hexosamines by the method of ELSON AND MORGAN[34] as adapted by RIMINGTON[35]. The amount of hexosamines was calculated as glucosamine from the standard curve. The results obtained are presented in Table IV.

As a significant difference was observed in the total hexose content before and after periodate oxidation of the sialic acid-free protein but not in the case of the original protein, the individual hexose constituents of the sialic acid-free protein and also of the original protein before and after periodate oxidation were determined in order to detect the hexose residues which were destroyed by periodate oxidation.

Identification and estimation of component hexoses by paper chromatography of proteins and sialic acid-free proteins before and after periodate oxidation

An aliquot of the hexose solution from the previous experiment was analysed for component hexoses by the descending paper-chromatographic method as adapted

by GEBHARDT[36] using Whatman No. 1 paper and the butanol – pyridine – water (6:4:3, v/v) solvent. The solvent was allowed to run off the paper for better separation of the hexoses. The chromatogram was dried at room temperature in a current of air, sprayed with aniline hydrogen phthalate reagent and then heated for 5 min at 105°. The sugars were identified with reference to the control chromatogram of known sugars run and developed under identical conditions. For the quantitative estimation of sugars, the method developed by JOSEPH AND BOSE[1] was followed. The spots were cut out, eluted with 5 ml of 50 % (v/v) aqueous dioxan at room temperature for 0.5 h and the absorbancy of the eluate was measured at 400 mμ in a Beckman DU spectrophotometer. The amount of sugar in each spot was calculated by reference to standard absorbancy plots obtained with known amounts of sugars run and developed under identical conditions. The results obtained are presented in Table IV.

TABLE IV

HEXOSE AND HEXOSAMINE CONTENTS OF SKIN PROTEINS AND SIALIC ACID-FREE PROTEINS BEFORE AND AFTER PERIODATE OXIDATION

Protein	*Periodate oxidation (h)*	*Total hexoses (%)*	*Total hexosamines (%)*	*Constituent hexose (%)*			
				Galactose	*Mannose*	*Glucose*	*Fucose*
Collagen	0	0.59	0.09	0.22	0.15	0.12	0.08
Collagen	5	0.59	0.08	0.21	0.16	0.11	0.08
Sialic acid-free collagen	0	0.61	0.08	0.23	0.16	0.12	0.08
Sialic acid-free collagen	5	0.47	0.08	0.10	0.15	0.11	0.09
Albumin	0	1.90	0.42	0.86	0.68	0.14	0.12
Albumin	5	1.85	0.27	0.83	0.65	0.14	0.11
Sialic acid-free albumin	0	1.93	0.43	0.88	0.69	0.13	0.12
Sialic acid-free albumin	5	1.38	0.29	0.45	0.65	0.12	0.12
Globulin	0	2.21	0.52	0.95	0.70	0.31	0.16
Globulin	5	2.23	0.35	0.94	0.68	0.32	0.16
Sialic acid-free globulin	0	2.24	0.52	0.97	0.72	0.30	0.17
Sialic acid-free globulin	5	1.57	0.34	0.36	0.69	0.29	0.18
Mucoid	0	6.97	1.69	3.81	1.72	0.90	0.38
Mucoid	5	6.88	1.09	3.75	1.70	0.90	0.39
Sialic acid-free mucoid	0	7.20	1.71	3.96	1.79	0.91	0.38
Sialic acid-free mucoid	5	4.61	1.12	1.47	1.75	0.92	0.36

DISCUSSION

It can be seen from Table I that of the skin proteins examined, the sialic acid content of mucoid is highest and that of citrate-soluble procollagen is lowest. The concentration of sialic acid in globulin is higher than in albumin. Elastin contains less sialic acid than collagen. WOOD[37] reported that the mucopolysaccharides are concerned with the nucleation and growth of collagen. BOWES *et al.*[38] suggested that insoluble collagen is a complex of citrate-soluble collagen and a mucoprotein. HIGHBERGER *et al.*[39] also reported that mucoprotein may be involved in the formation of collagen fibres *in vivo*. In the present investigation, no sialic acid could be detected in neutral-salt-soluble collagen while citrate-soluble collagen contains much less than the mature collagen, which also indicates that the added fraction may be a mucopolysaccharide-containing sialic acid.

It is also clear that in the case of collagen, procollagens or elastin, there is good

agreement between the results obtained by the thiobarbituric acid and the resorcinol methods, whereas in the case of globular proteins the values obtained by the resorcinol method are somewhat higher. The results presented in Table II show that the NANA is probably the only form of sialic acid present in collagen, procollagens and elastin whereas both NANA and NGNA occur in skin albumin, globulin and mucoid; NGNA constituting only 24.1, 20.2 and 9.6 % of their total sialic acid content, respectively. WARREN[24] reported that in the resorcinol method the molar extinction coefficient of NANA is 16 % lower than that of NGNA whereas in the thiobarbituric acid method the molar extinction coefficient of NGNA is 19 % lower than that of NANA. Thus the difference between the values obtained by the resorcinol and thiobarbituric acid methods may arise in the case of globular proteins which contain NGNA, because the calculation of results is based upon the molar extinction coefficient of NANA only.

The possibility of the presence of minor amounts of other forms of sialic acid, such as the *N,O*-diacetyl derivatives in skin proteins, however, is not completely ruled out. As mild acid treatment is known to convert *N,O*-diacetylneuraminic acid to NANA, such a conversion from *N,O*-diacetyl form to *N*-acetyl derivative may take place during acid hydrolysis and hence the detection of *N,O*-diacetyl derivative may not be possible even though it was originally present in the untreated protein.

Table III shows that about 27–35 % of the total sialic acids of these proteins are liberated after 0.5-h hydrolysis and about 71–80 % after 4-h hydrolysis by neuraminidase action. With prolonged incubation periods and repeated action with higher concentration of neuraminidase, it was possible to release essentially all the sialic acids from collagen, albumin, globulin and mucoid (Table IV). These observations indicate that all of the sialic acid moieties of these proteins are not equally available to neuraminidase action. Though sialic acids possess strong reducing power, none of these proteins was found to reduce Benedict's reagent under standard conditions. These observations suggest that the reducing group of the sialic acid of these proteins is joined terminally in an *O*-glycosidic linkage to the adjacent unit.

It can be seen in Table IV that fresh hexosamine residues are not exposed or available for periodate oxidation after removal of the sialic acids from collagen, albumin, globulin and mucoid. Thus a linkage of sialic acids to the hexosamine residues in these proteins seems to be excluded. On the other hand, the hexose residues have been found to be susceptible to attack by periodate only after the enzymic removal of sialic acids from these proteins. It has also been observed that only the galactose content of each of the sialic acid-free proteins is appreciably lower after periodate oxidation than that before periodate oxidation but there is no significant difference in the galactose content of the original protein before and after periodate oxidation. These observations suggest that the galactose residues are exposed for periodate oxidation only after the removal of sialic acids and hence galactose is the major partner for the *O*-glycosidic linkage of sialic acids in these proteins. It is interesting to note that the galactose content in each of these proteins is higher than any other hexose or hexosamine.

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