440. *Frog-spawn Mucin.*

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Frog-spawn mucin, which contains some 42% of carbohydrate, on hydrolysis yields L-fucose (7·1%), D-mannose (3·5%), D-glucose (?), xylose (?), D-galactose (12·7%), D-glucosamine (8·9%), and D-chondrosamine (9·5%). Reaction of the mucin with periodate results in the destruction of the fucose and of some of the amino-sugars. Eighteen amino-acids have been identified after hydrolysis of the mucin, and the amounts determined quantitatively.

The analysis of the mucins (protein polysaccharide complexes) has been delayed by the lack of suitable methods for the quantitative determination of amino-acids and sugars. For example, the separation and identification of the sugars produced on hydrolysis is a matter of some difficulty owing to the interfering effect of the amino-acids and peptides produced simultaneously. The development of new methods (Hough, Jones, and Wadman, *J.*, 1949, 2511) has facilitated the separation of the sugars and we now report the results of an examination of the sugars and amino-acids produced on hydrolysis of frog-spawn mucin (cf. Giacosa, *Z. physiol. Chem.*, 1882, **7**, 40; Schulz and Ditthorn, *ibid.*, 1900, **29**, 373). This material is known to contain D-galactose (van Ekenstein and Blanksma, *Chem. Weekblad*, 1917, **4**, 407; Pirie, *Brit. J. Exp. Path.*, 1936, **17**, 273; cf. Bray, Henry, and Stacey, *Biochem. J.*, 1946, **40**, 124) and fucose (Bray and James, 1st International Congress of Biochem., Abs. 267/6, 225) and it was of interest to determine whether L-galactose was also present as had been observed in snail galactogen (Baldwin and Bell, *J.*, 1938, 1461; 1941, 125). A preliminary examination of the products of hydrolysis of the mucin showed the presence of fucose, galactose, and glucosamine, provisionally identified on the paper chromatogram by the method of Partridge (*Biochem. J.*, 1948, **42**, 238). In order to confirm the identification of these sugars the mucin was hydrolysed, the acid and acidic amino-acids were removed with Amberlite resin IR4B, and the residual neutral and basic compounds separated on a column of cellulose, *n*-butanol half-saturated with water being used as eluent. In this manner the following sugars were isolated.

(*a*) L-Fucose was identified as the crystalline sugar and as its characteristic crystalline toluene-*p*-sulphonylhydrazone. The optical rotation of the crude sugar (−68°) showed that either some D-fucose or some other more positively rotating material was present. As the fraction gave a positive ninhydrin reaction this may have been amino-acid. *N*-Acetylglucosamine moves at the same rate as fucose on the chromatogram and may have been a contaminant.

(*b*) D-Mannose, was isolated as the characteristic phenylhydrazone. A solution of the phenylhydrazone in dilute hydrochloric acid furnished D-mannose, which behaved on the chromatogram in a manner identical with that of an authentic specimen of D-mannose phenylhydrazone treated in a similar fashion. This sugar and D-glucose were not detected in the preliminary hydrolysis experiments owing to their low concentration. Separation of the sugar mixture on a column, however, leads to concentration of the sugars, which can then be detected.

(*c*) D(?)-Glucose was identified as its osazone and by its rate of movement on the chromatogram. The low rotation of this fraction (+27°) indicated that other material, some of which was amino-acid, was also present.

(*d*) D-Galactose was isolated as the crystalline sugar. No L-galactose could be detected.

(*e*) D-Glucosamine hydrochloride. The identity of this material was confirmed by its conversion into D-glucosazone and by the identity of its *X* ray photograph with that of an authentic specimen.

(*f*) D-Chondrosamine was isolated as its crystalline hydrochloride which, with phenylhydrazine and acetic acid, gave galactosazone. The *X*-ray photograph was identical with that of an authentic specimen of chondrosamine hydrochloride. This amino-sugar was an unexpected component of the sugar mixture (cf., however, Schmidt, "Organic Chemistry," 5th edn., p. 812). Its separation from glucosamine on the chromatogram is unreliable and it escaped detection in the early experiments. Fucose, galactose, glucosamine, and chondrosamine have been detected in frog-spawn mucin by Drs. H. G. Bray and S. P. James (private communication). Xylose may also be present (see p. 2139).

The positions of the sugars on the chromatogram were revealed by spraying with *p*-anisidine hydrochloride solution in *n*-butanol (Hough, Jones, and Wadman, *J.*, 1950, 1702). The sites of the amino-sugars were shown by spraying duplicate papers with ninhydrin solution or Ehrlich's reagents. The yields of sugars obtained from the separation on the column of cellulose enabled an estimate of the percentages of the sugars present in the original mucin to be made. A more accurate determination of the sugars was made by analysis of the products of hydrolysis

of the mucin by the method of Flood, Hirst, and Jones (*J.*, 1948, 1679) as modified by Hirst and Jones (*J.*, 1949, 1659). The methyl pentose content of a sample of hydrolysed mucin was also determined by the method of Nicolet and Shinn (*J. Amer. Chem. Soc.*, 1941, **63**, 1456) and was found to be in close agreement with the fucose percentage determined as described above. The total amino-sugar content of a sample of hydrolysed mucin was checked colorimetrically by the method of Vasseur (*Arkiv Kemi*, 1949, **1**, 253): L-fucose, 7·1% (calc. as $C_6H_{10}O_4$), D-mannose, 3·5% (calc. as $C_6H_{10}O_5$), D-galactose, 12·7% (calc. as $C_6H_{10}O_5$), D-glucosamine, 8·9% (calc. as $C_6H_{11}O_4N$), and D-chondrosamine, 9·5% (calc. as $C_6H_{11}O_4N$).

The mucin was oxidised with sodium periodate solution with the resultant destruction of all the fucose and of some of the amino-sugar. During the oxidation formic acid was produced in amount corresponding to the formation of approximately one mol. of acid per mol. of fucose, indicating that this sugar is in the pyranose form and that it is an end group. The majority of the galactose appeared to be unaffected, and some of this fraction is therefore probably linked either through $C_{(3)}$ or through $C_{(2)}$ and $C_{(4)}$. The identity of the unoxidised amino-sugars remains to be determined. The amino-sugars are present very probably as *N*-acetyl derivatives (Found: *N*-acetyl, 9·4; this figure is high and other volatile acids may be present). The above evidence indicates that some 50% of the amino-sugar (if in the pyranose form) is present as end group, or is linked through $C_{(1)}$ and $C_{(6)}$ only. The glucose detected on the chromatogram may be part of the carbohydrate moiety of the mucin or it may result from the hydrolysis of a small amount of glycogen present as impurity. The method of isolation of the mucin, involving as it does a treatment of the frog spawn with 0·01N-sodium hydroxide at 35—40°, may result in the liberation of some of the glycogen which is present in the embryo. The mucin gave no detectable intensification of colour with aqueous iodine; the amount of glycogen admixed with the mucin must therefore be small. The carbohydrate moiety resembles the carbohydrate material obtained from other sources by Bray, Henry, and Stacey (*loc. cit.*) and by Aminoff, Morgan, and Watkins (*Biochem. J.*, 1950, **46**, 426). The latter authors have reported the presence of hexosamine, galactose, and fucose in the molecular proportions 2 : 1 : 1 in blood group A substance isolated from pseudomucinous ovarian cyst fluid. No mannose was detected.

The amino-acid content of the mucin was determined after hydrolysis with hydrochloric acid or sodium hydroxide. The majority of the amino-acids, provisionally identified by two-dimensional chromatography (Dent, *Biochem. J.*, 1948, **43**, 169), were determined by a modification (Folkes and Yemm, to be published) of the microbiological assay of Henderson and Snell (*J. Biol. Chem.*, 1948, **172**, 15). Alanine and threonine were determined by colorimetric estimation of the acetaldehyde produced on oxidation with ninhydrin (Virtanen and Rautanen, *Biochem. J.*, 1947, **41**, 101) and periodic acid (Winnick, *J. Biol. Chem.*, 1942, **142**, 461), respectively. Colorimetric tests (McFarlane and Guest, *Canadian J. Res.*, 1939, *B*, **17**, 139) for hydroxyproline yielded negative results. Amide nitrogen was determined by dilute acid hydrolysis followed by colorimetric estimation of the ammonia produced (Yemm and Folkes, to be published).

The results are given in the table (p. 2140). The amino-sugars, amino-acids, and amides account for nearly 94% of the total nitrogen. The threonine estimate may be slightly high, since traces of L-fucose which might have survived the severe hydrolysis conditions employed would also yield acetaldehyde on periodate oxidation. The cystine and methionine, as determined, contain only 90% of the total sulphur. The difference is probably due to the destruction of cystine during hydrolysis.

The ammonia content (12·4% of the total N) of the acid hydrolysate is much higher than would be expected from the amide content (6·3% of the total N), even after allowance for slight destruction of the hydroxyamino-acids. Experiments with glucosamine have shown that *ca.* 21% of its nitrogen is liberated as ammonia when it is subjected to the conditions employed to hydrolyse the mucin. If the same rate of destruction of the amino-sugars operates during the hydrolysis of the mucin, then some 4% of its total nitrogen would be liberated as ammonia.

The physical properties of the mucin and the comparatively small amount of material available rendered attempts to determine its homogeneity unsatisfactory. The results quoted above are, therefore, given with reserve, as more than one mucin or protein may be present. Moreover, some 10—15% of the mucin has not been identified. Some of this may be solvent as it has been found very difficult to remove the last traces of moisture even under reduced pressure at 100°. It is apparent, however, that the carbohydrate portion differs from the plant gums and mucilages in that it contains hexosamine residues. No uronic acid was detected No evidence is available as to the mode of linkage of the carbohydrate and protein portions of the mucin, nor can much be said about the mode of linkage of the sugars, except that

fucose is an end group and that some of the galactose is linked through at least two carbon atoms.

In a private communication, Drs. H. G. Bray and S. P. James, of Birmingham University, inform us that they have also examined the hydrolysis products of a mucin obtained from frog spawn by dissection. The carbohydrate moiety appears to contain chondrosamine as the major amino-sugar component, along with galactose and fucose. Scission of the mucin with 0·05N-sodium carbonate at 100° for 5 minutes liberates a portion of the amino-sugar. Serine, glycine, threonine, alanine, lysine, proline, valine, leucine, aspartic acid, glutamic acid, arginine, cysteine (as cysteic acid) and phenylalanine were also detected in the acid hydrolysis products of the mucin by paper partition chromatography. Methylation of a mucin obtained by extraction of the spawn with barium hydroxide yielded an ether-soluble methylated product (A) which contained end groups of L-fucopyranose (7%) and D-galactopyranose (7%), identified as crystalline 2 : 3 : 4-trimethyl α-methyl-L-fucoside and 2 : 3 : 4 : 6-tetramethyl D-galactose anilide, respectively. Other partly methylated sugars await identification, but no amino-sugar derivatives were detected. The methylated derivative (A) probably represents a highly degraded fraction of the mucin.

It is apparent that much further work will be required to elucidate the structure of this mucin.

Experimental.

Isolation of the Mucin.—Fresh frog spawn was washed and separated as completely as possible from water and organic debris. N-Sodium hydroxide was then added with stirring until the total concentration was 0·01N. relative to the volume of the frog spawn. The mixture of spawn and alkali was then warmed to 35—40°; the outer jelly then rapidly dissolved, allowing the inner black embryo to settle out. The alkaline solution of mucin was decanted from the embryos and immediately made slightly acid by addition of acetic acid. The mucin was precipitated by pouring it into methanol (5 vols.), separated by decantation, washed by decantation with methanol and then with acetone, and collected on a filter. It was dried under reduced pressure at room temperature, and was obtained as a white powder insoluble in cold water, soluble in sodium hydroxide [Found: C, 40·6; H, 6·8; N, 8·1 (Dumas), 8·0 (Kjeldahl); S, 0·95; *N*-acetyl, 9·4; sulphated ash, 3·6%, on a sample dried at 100°/16 mm.].

Analysis of the Mucin.—The dried mucin (57 mg.) was dissolved in hydrochloric acid (2N.) and hydrolysed in a sealed tube at 100° for 7 hours. The tube was then cooled and opened, and the solution evaporated to a syrup under reduced pressure in order to remove most of the hydrochloric acid. The solution was then neutralised and the volume adjusted to 20 c.c. The amino-sugar content was determined colorimetrically on 4 c.c. of solution by the method of Vasseur (*loc. cit.*) (Found: 2·91 mg. of amino-sugar hydrochloride. This corresponds to the presence of 21% of amino-sugar, calculated as $C_6H_{11}O_4N$ and chondrosamine and glucosamine being assumed to give the same colour intensity per unit weight).

The mucin (34·2 mg.) was suspended in N-hydrochloric acid (10 c.c.), and the mixture heated on the boiling-water bath. At intervals, portions (1 c.c.) were withdrawn and the reducing power determined by the method of Hirst, Hough, and Jones (*J.*, 1949, 928) [Found: mg. of hexose liberated per c.c., 1·07 (35 minutes); 1·46 (2¾ hours); 1·43 (3¾ hours)]. This correspond to the liberation of 14·3 mg. of hexose or 37·6% of reducing sugar (as $C_6H_{10}O_5$). The solution was heated for a further 8 hours.

To the remaining solution (6 c.c.; equivalent to 20·5 mg. of mucin), rhamnose hydrate (4·17 mg.) was added and the cooled solution was neutralised with Amberlite resin IR4B and filtered. The filtrate and washings were evaporated to a syrup, and the sugars separated chromatographically on paper in the usual manner (Found, in duplicate experiments: rhamnose hydrate, 0·273, 0·300; fucose, 0·111, 0·111; mannose, 0·054, 0·054; galactose, 0·187, 0·210; glucosamine as the hydrochloride, 0·163, 0·170; chondrosamine as the hydrochloride, 0·174, 0·182 mg.). These figures correspond to the presence of fucose, 7·4, 6·7% (calc. as $C_6H_{10}O_4$), mannose, 3·6, 3·3%, galactose, 12·6, 12·8% (both calc. as $C_6H_{10}O_5$), glucosamine, 9·1, 8·6%, and chondrosamine, 9·7, 9·2% (both calc. as $C_6H_{11}O_4N$). No great accuracy is claimed for these last two pairs of figures owing to the difficulty in separating completely the two amino-sugars on the chromatogram.

Determination of Methyl Pentose (Nicolet and Shinn, *loc. cit.*).—The dried mucin (78·1 mg.) was hydrolysed with 0·5N-sulphuric acid (5 c.c.) for 7 hours. The solution was neutralised with solid sodium hydrogen carbonate, and the acetaldehyde determined after oxidation with periodate solution (Found: acetaldehyde equivalent to 7·17 c.c. of 0·01N-thiosulphate solution, *i.e.*, 6·8% of fucose calc. as $C_6H_{10}O_4$).

Hydrolysis of the Mucin and Isolation of the Sugars produced—Method A. The mucin (1·082 g.) was suspended in water (40 c.c.), and N-hydrochloric acid (40 c.c.) added. The mixture was then heated on the boiling-water bath (temp. of solution, 87°) for 26 hours. The cooled solution, which was pale yellow, was filtered free from humic material, and the filtrate passed down a column of IR4B resin. The neutral effluent and washings were concentrated to a brownish syrup, which was dissolved in methanol (5 c.c.), and the solution placed on top of a column of hydrocellulose (1½" × 12"). *n*-Butanol, half-saturated with water, was then allowed to percolate down the column, and the effluent was collected automatically in small fractions (*ca.* 6 c.c.). The detection and identification of the sugars in the eluate were carried out by the method of Hough, Jones, and Wadman (*loc. cit.*). Complete separation of all the sugar fractions was attained.

The solvent was removed from each fraction by distillation under reduced pressure at 50°, and the residue dissolved in water, filtered, and the filtrate concentrated under reduced pressure. Six fractions were obtained.

(1) This fraction was obtained as a syrup which slowly crystallised. Determination of the sugar content showed the presence of 59 mg. of reducing material calculated as fucose, $[\alpha]_D$ −68° (*c*, 0·59 in water) (yield, 4·9%, calc. as $C_6H_{10}O_4$). The syrup was triturated with methanol and a sample of crystalline L-fucose (3 mg.), m. p. and mixed m. p. 144°, was obtained, $[\alpha]_D$ −82° ± 6° (*c*, 0·4 in water). The methanolic filtrate was warmed with an alcoholic solution of toluene-*p*-sulphonylhydrazine, and L-fucose toluene-*p*-sulphonylhydrazone was precipitated (72 mg.). It was recrystallised from methanol and had m. p. and mixed m. p. 175°. The low optical rotation of the crude sugar may be due to the presence of D-fucose or xylose (?). Some material which gave a positive ninhydrin reaction was present.

(2) Estimation of the sugar content of Fraction 2 showed the presence of mannose (16·4 mg.), $[\alpha]_D$ +14° (*c*, 0·14 in water) (yield, calc. as $C_6H_{10}O_5$, 1·4%). This sugar did not crystallise. Phenylhydrazine acetate solution (1 c.c.) was added to the syrup, and the solution set aside. After two days the supernatant liquid was withdrawn from the crystals of mannose phenylhydrazone which adhered to the bottom of the flask. The crystals were washed successively with water, alcohol, and ether and dried (m. p. and mixed m. p. 185°, decomp.). A sample of the phenylhydrazone was dissolved in a drop of warm hydrochloric acid (2N.), and the solution placed on a chromatogram. A sample of mannose phenylhydrazone was treated in the same fashion. After separation in the usual manner it was found that the sugar liberated from the phenylhydrazone had moved through the same distance as had mannose, and that it gave the same colour reactions when the chromatogram was heated after it had been sprayed with aniline trichloroacetate or *p*-anisidine hydrochloride.

(3) This fraction (15 mg., from the determination of reducing power) did not crystallise; $[\alpha]_D$ +27° (*c*, 0·15 in water). When its solution was heated with phenylhydrazine acetate solution, glucosazone was formed, identified by its characteristic crystalline shape. The syrup gave a positive ninhydrin reaction and may have contained amino-acid as well as sugar.

(4) Determination of the sugar content of this solution showed the presence of 148 mg. of material (12·3%), $[\alpha]_D$ +79° (*c*, 1·2 in water). On concentration, crystalline D-galactose (128 mg.) was obtained, m. p. and mixed m. p. 165°.

(5) The solution obtained on concentration of this fraction was too dark for polarimetric observations. On standing, crystals of glucosamine hydrochloride separated. These were filtered off, washed with methanol, and dried (yield 108 mg., 7·5%, calc. as $C_6H_{11}O_4N$), $[\alpha]_D^{20}$ +72° (*c*, 1·0 in water). When a sample was heated with phenylhydrazine acetate solution, characteristic crystals of glucosazone were produced. An *X*-ray photograph of the crystals of the hydrochloride was identical with that obtained from a sample of authentic glucosamine hydrochloride.

(6) The solution obtained on concentration of this fraction was brown, and polarimetric observation was not possible. Concentration of the solution gave crystalline chondrosamine hydrochloride, which was separated by trituration with methanol, $[\alpha]_D^{20}$ +109° (*c*, 1·0 in water) (yield 93 mg., 6·4%, calc. as $C_6H_{11}O_4N$). A specimen of this material when heated with phenylhydrazine acetate solution gave galactosazone (characteristic crystals and solubility). An *X*-ray photograph of the crystalline amino-sugar hydrochloride differed markedly from that obtained from glucosamine hydrochloride (cf. Cutler, Haworth, and Peat, *J.*, 1937, 1979), and was identical with that obtained from authentic chondrosamine hydrochloride (Stacey, *J.*, 1944, 272).

Method B. The mucin (0·74 g.) was boiled with methanolic hydrogen chloride (50 c.c.; 2%) for 16 hours. The cooled solution was filtered, and the filtrate neutralised with silver carbonate and again filtered. The solution of glycosides and of esters of amino-acids was concentrated to a syrup, and the residue dissolved in water. When the solution was percolated down a column of IR100H Amberlite resin, practically all the amino-acid derivatives were removed. The eluate was concentrated to a syrup, which was dissolved in N-sulphuric acid (50 c.c.), and the dark solution heated on the boiling-water bath for 12 hours. The cooled solution was exactly neutralised with barium hydroxide and filtered. Concentration of the filtrate gave a syrupy residue (0·20 g.), which was separated on a column of cellulose, *n*-butanol half saturated with water being used as eluent. This led to the isolation and identification of samples of L-fucose and D-mannose. Xylose (5 mg.) was provisionally identified as its dibenzylidene dimethyl acetal, m. p. 205°, by its rate of movement on the chromatogram, and by the red colour produced by it on the chromatogram when warmed with aniline phthalate. The xylose may be an artefact and not a component of the mucin. No uronic acid was present (naphtharesorcinol test).

Oxidation of the Mucin by Periodate.—(a) The mucin (551 mg.) was suspended in water (50 c.c.), and 0·01N-sodium hydroxide (7 c.c.) added to make the solution neutral to "screened" methyl-red. The solution was cooled to 0°, and sodium periodate solution (7 c.c., 0·5M.) added. The solution was kept at 2° in the dark for 30 hours. (The oxidation of starch proceeds to completion under these conditions; Halsall, Hirst, and Jones, *J.*, 1947, 1399.) Ethylene glycol was added, and the solution titrated with 0·1N-sodium hydroxide (Found: 4·01 c.c., corresponding to the production of one mol. of formic acid per mol. weight 1380). This amount of formic acid would be produced from 11·8% of hexose end group (calc. as $C_6H_{10}O_5$) or from 10·6% of methyl pentose end group (calc. as $C_6H_{10}O_4$).

(b) The mucin (226·3 mg.) was oxidised with sodium periodate solution (10 c.c.; 0·5M.) as described above. Excess of periodate was removed by addition of ethylene glycol, and the solution dialysed until free from iodate ions. The solution was concentrated under reduced pressure, and a portion (115 mg.) of the pale yellow solid residue (140 mg.) hydrolysed in a sealed tube with N-hydrochloric acid at 100° for 7 hours. The volume was adjusted to 10 c.c. A portion (2 c.c.) of the cooled solution was neutralised with Amberlite resin IR4B, and the solution filtered and concentrated to a syrup. Examination of the syrup on the chromatogram showed the absence of fucose and the presence of a trace

of mannose and glucose. An intense spot corresponding to the presence of galactose was detected. On spraying with Ehrlich's reagents two spots corresponding to glucosamine and chondrosamine were detected, the former of lower intensity than the latter. Colorimetric determination on a portion of the solution showed the presence of *ca.* 11% of amino-sugar (calc. as $C_6H_{11}O_4N$).

Amino-acid Determinations.—The mucin (458 mg.) was heated in a sealed tube and in an autoclave at 15 lb. pressure with 3N-hydrochloric acid (10 ml.) for 5 hours. The solution was filtered to remove humin material and diluted to about 25 ml., the pH adjusted to 6·0 with sodium hydroxide, and the volume brought to 50 ml. with water. The solution was stored under toluene in a refrigerator until required. Total-nitrogen determinations (Kjeldahl) on 1-ml. aliquots showed 717, 718 μg. N/ml. [Calc. from total N of mucin (Kjeldahl 7·96, 7·98%): 731 μg. N/ml.]. The difference is assumed to be due to nitrogen bound in the humin material (lost during Kjeldahl determination). Free ammonia of 2-ml. aliquots was 90·1, 90·4 μg. N/ml. (12·35% of total N).

All the amino-acids estimations (with the exception of tryptophan and hydroxyproline) were carried out on this hydrolysate. Details of the methods employed are to be published elsewhere. Sixteen of the eighteen amino-acids were determined by a modification of the microbiological assay of Henderson and Snell (*loc. cit.*), *Leuconostoc mesenteroides* and *Lactobacillus arabinosus* being used as the assay

Amino-acid calc. as:	Amino-acid, mg., per ml. of hydrolysate.	Total N, %.
Acid hydrolysate (total N, 731 μg./ml.).		
Alanine	0·142	3·1
Arginine (HCl)	0·166	6·1
Aspartic acid	0·359	5·2
Cystine	0·118	1·9
Glutamic acid	0·363	4·7
Glycine	0·474	12·1
Histidine (H_2O, HCl)	0·101	2·8
*iso*Leucine	0·225	3·3
Leucine	0·236	3·5
Lysine (HCl)	0·192	4·0
Methionine	0·209	2·7
Phenylalanine	0·125	1·5
Proline	0·325	5·4
Serine	0·210	3·8
Threonine	0·174	2·8
Tyrosine	0·137	1·5
Valine	0·154	2·5
Alkaline hydrolysate (total N, 185·5 μg./ml.).		
Tryptophan	0·0081	0·6
Amide N	—	6·3
Amino-sugars	—	20·0
	Total	93·8

organisms. All assays were carried out in triplicate at each of five levels of unknown and standard. Results are expressed in the table as mg. of amino-acid found per ml. of hydrolysate and as the calculated percentage of total nitrogen corresponding to each amino-acid.

Alanine was determined by oxidation with ninhydrin (Virtanen and Rautanen, *loc. cit.*) followed by estimation of the acetaldehyde produced by a modification of Stotz's *p*-hydroxydiphenyl colour reaction (*J. Biol. Chem.*, 1943, **148**, 585). Winnick's method (*loc. cit.*) was followed for the threonine estimations, *viz.*, oxidation with periodic acid in Conway micro-diffusion units and determination of acetaldehyde by the *p*-hydroxydiphenyl method.

Determination of Tryptophan. (With Miss J. C. Hillman).—The mucin (93·2 mg.) was hydrolysed by 17 hours' autoclaving in a sealed tube with 5N-sodium hydroxide (1 ml.). After adjustment with hydrochloric acid to pH 6·0, the volume was made up to 40 ml. (Calc. total N, 185·5 μg./ml.). Tryptophan was determined by microbiological assay, *L. arabinosus* being used. Complete racemisation was assumed to have taken place during hydrolysis, and DL-tryptophan was employed as the standard.

Test for Hydroxyproline.—A hydrolysate was prepared by heating 200 mg. of the mucin with 3N-sulphuric acid (2 ml.) in a sealed tube at 100° for 12 hours. After neutralisation, the solution was filtered and made up to 5 ml. 1-Ml. aliquots were employed for the colour test (McFarlane and Guest, *loc. cit.*) but no hydroxyproline could be detected; neither was it detected on the chromatogram.

Determination of Amide Nitrogen.—Weighed portions of the mucin (16·05, 13·50 mg.) were hydrolysed with 2N-sulphuric acid (10 ml.) at 100° for 3 hours. The ammonia produced was distilled off and determined by Nesslerisation (Found: N, 796, 683 μg., corresponding to an amide content of 6·23, 6·35% of total N).

Destruction of Glucosamine on Acid Hydrolysis.—D-Glucosamine hydrochloride (70 mg.) was autoclaved in a sealed tube at 15 lb. pressure for 5 hours with 3N-hydrochloric acid (2 ml.). The solution was diluted to 10 ml., and 1 ml. aliquots were taken for the determination of free ammonia (Found: 92, 96 μg. ammonia N/ml., equivalent to 20, 21% of the total nitrogen of the glucosamine).

The authors thank Dr. T. Malkin for the *X*-ray photographs of glucosamine and chondrosamine hydrochloride, and Mr. B. Clist for carrying out the colorimetric determination of amino-sugars.

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[*Received, May 24th*, 1950.]