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NEURAMINIC ACID: THE FUNCTIONAL GROUP OF SOME BIOLOGICALLY ACTIVE MUCOPROTEINS

Present knowledge would attribute to proteins a wider range of biological activity than is possessed by any other group of natural substances. Thus enzymes, respiratory pigments, antibodies, many antigens, and several hormones are protein in nature. Sometimes the biologically active substance is a simple protein, consisting of amino acid residues only (papain, carboxypeptidase, lysozyme, insulin, glucagon, β-corticotropin, etc.). In other cases the protein is conjugated with nonprotein material as in the photosynthetic pigments, respiratory enzymes, the oxygen carriers haemoglobin and chlorocruorin, the gonadotropic hormones, Castle's intrinsic factor, blood group substances, virus haemagglutinin inhibitors, and others. Whereas it is well established that the haem iron in haematoproteins provides the active centre, and that flavin adenine mononucleotide and dinucleotide, respectively, are the functional groups of catalytically active flavoproteins, the literature provides little, if any, information about the structural element responsible for the biological activity of mucoproteins, i.e., carbohydrate-protein complexes with high protein content.

Over the past nine years the staff of this Institute has been engaged in an investigation of the interaction between the influenza virus particle and some mucoproteins at both the biological and the biochemical levels. These investigations, together with those from other laboratories, resulted in a definition of the biological activity of the mucoproteins concerned in the isolation of the functional component and in the elucidation of its chemical structure.

The various observations, in their chronological order, leading to the information that certain mucoproteins display biological activity when influenza virus is used as indicator system, have been described in detail in a previous paper[15] in this Journal. The results up to that time and their interpretation may be summarized as follows:

1. Mucoproteins prepared from human urine (UM) and from bovine submaxillary glands (BSM) in an electrophoretically homogeneous state inhibit in high dilution haemagglutination by appropriately heat-treated

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virus particles (indicator virus) of certain strains. This holds also for the mucoprotein fraction ovomucin prepared from egg-white.[6]

2. When pretreated with living influenza virus or with a purified soluble enzyme (RDE) from vibrio cholerae, these mucoproteins lose irreversibly the ability to inhibit virus haemagglutination.[5]

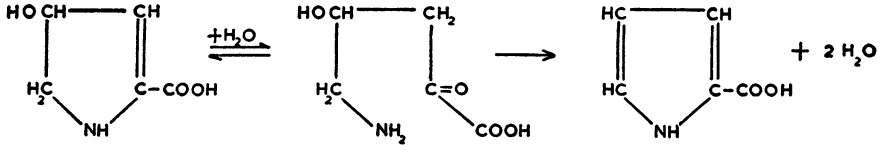
3. Concomitant with the loss of biological activity, a small molecular weight compound is released from the mucoprotein upon virus or RDE action.[22]

These observations indicated the operation of attractive forces between heated virus and the soluble mucoprotein resulting in adsorption of the virus particle to the fibrous mucoprotein and thus preventing haemagglutination. A further inference from the findings was the presence of an enzymatically active grouping at two or more sites of the virus surface attacking the inhibitory mucoprotein in such a way as to split off a small fragment of the molecule and, by doing so, to deprive the mucoprotein of its biological activity. Obviously, then, the biological activity of the mucoproteins towards the influenza virus resides in or is closely associated with the enzymatically liberated split product, and it seemed of great interest to find out the composition and structure of this segment of the mucoprotein.

As already mentioned in the earlier review,[15] the split product exhibited quite unusual features. It had reducing power and showed several reactions characteristic for carbohydrates, but decomposed with humin formation when treated with 1.0 N HCl for 30 minutes at 100° C. It contained nitrogen and after treatment with 0.1 N sodium carbonate for 12 minutes at 100° C. it coupled with the Ehrlich reagent (p-dimethylaminobenzaldehyde in hydrochloric acid) to give a purple color which spectrophotometrically was identical with that produced by an authentic specimen of N-acetyl D-glucosamine. In contrast to N-acetylglucosamine, however, the chromogen was alkali stable and after treatment with 1.0 N HCl at 100° for 60 minutes it failed to give the reaction of Elson and Morgan[9] shown by N-acetylhexosamine after acid de-acetylation. Odin,[32] confirming our results, added that the split product gave the Ehrlich reaction even without pretreatment with alkali (direct Ehrlich reaction) and drew attention to the identity, in their general reactions, of the split product and of sialic acid, a reducing acid of the probable composition $C_{13}H_{23}O_{11}N$ or $C_{13}H_{21}O_{10}N \cdot H_2O$ but of unknown structure,[2] first isolated by Blix[1] from bovine submaxillary gland mucin.

A major step towards the elucidation of the structural chemistry of the enzymatically released compound was taken when 2-carboxy-pyrrole was isolated[14, 17] from the alkali hydrolysate of homogeneous inhibitory mucoproteins (UM and BSM). The 2-carboxy-pyrrole, like the split product,

reacts with Ehrlich reagent directly, i.e., without alkali pretreatment and, being unstable towards mineral acid, loses its chromogenic quality on treatment with acid. Our assumption that 2-carboxy-pyrrole was present as such in the inhibitory mucoproteins and in the split product was not borne out by further work. The high value of the molecular extinction coefficient of 2-carboxy-pyrrole ($\epsilon = 11700$ in phosphate buffer, pH7.0) at a wavelength ($\lambda_{max} = 256$ mμ), where the aromatic amino acids have a low extinction coefficient, would have rendered it possible to detect any preformed 2-carboxy-pyrrole in the native mucoprotein and in the split product. Contrary to expectation neither the undegraded mucoprotein nor the untreated split product exhibited absorption at 256 mμ. However, when the split product was treated with 0.1 N sodium carbonate for 20 minutes at 100°, a substance could be extracted with ether from the acidified solu-



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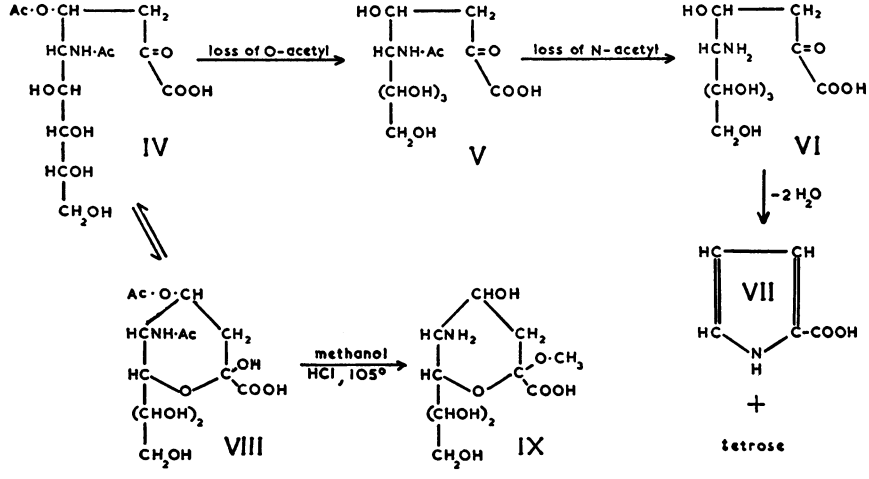
tion which on spectrophotometric and chromatographic analysis proved to be identical with synthetic 2-carboxy-pyrrole.[16,17] These findings suggested the presence in the mucoprotein and in the split product of a structure readily converted to 2-carboxy-pyrrole under very mild alkaline conditions. Since glutamine, glutamic acid, 2-carboxy-5-pyrrolidone, proline, hydroxyproline, N-acetylglucosamine under mild alkaline conditions did not yield 2-carboxy-pyrrole, it was postulated[16] that the immediate precursor of 2-carboxy-pyrrole is the unstable 2-carboxy-4-hydroxypyrroline (I) which in aqueous solution will undergo reversible ring-opening. The open-chain product (II) as a β-oxyketone would be unstable to alkali (or acid) and reclosure of the ring will result in the formation of 2-carboxy-pyrrole (III). It was further assumed[16,17] that within the framework of the native mucoprotein, and in the split product, I is prevented from spontaneous rearrangement to III by substitution at the hydroxyl group (R for H). Removal by alkali or acid of the substituent R will induce production of III.

Recent developments have both substantiated and qualified this concept. The coincidence in their general properties of sialic acid from BSM and of the split product from BSM and UM and our further observation[18] that along with the enzymatic release of the split product the sialic acid content

of BSM was reduced by 64 p.c., as estimated spectrophotometrically, suggested close structural relationship, if not identity, of the two compounds and, by implication, marked sialic acid as the component of BSM giving rise to the formation of 2-carboxy-pyrrole upon alkali treatment of this inhibitor. Blix, Lindberg, Odin, and Werner[2] found sialic acid prepared from BSM to contain an N-acetyl group, an O-acetyl group, which is very easily split off, a primary alcohol group, an α-hydroxy group, and a total of five hydroxyl groups. On the basis of these data, its empirical formula $C_{13}H_{21}O_{10}N : H_2O$, its general properties, and its convertability to 2-



carboxy-pyrrole we have assigned[18] to sialic acid the provisional structure IV. Obviously, IV by loss of its two acetyl groups would form VI, i.e., a delta-substituted II, which in turn would cyclise to the pyrrole VII with elimination of a tetrose. The susceptibility to acid and alkali of the ester and amide linkages joining the two acetyl groups to the rest of the sialic acid molecule would account for the formation of 2-carboxy-pyrrole and for the direct Ehrlich reaction given by sialic acid and the split product.

An important contribution bearing on the composition of the split product was made by Klenk and collaborators. In 1951 Klenk[24] isolated from gangliosides the methoxy derivative of neuraminic acid, and recently the same compound was prepared from bovine submaxillary gland mucin and from the inhibitory urine mucoprotein.[28] This compound has no reducing power, is ninhydrin positive, and is stable to alkali; therefore, it does not give the Ehrlich reaction in the cold after pretreatment with alkali. However, when heated in mineral acid, the methoxy derivative of neuraminic acid resembles sialic acid and the split product in the colours formed

with p-dimethylaminobenzaldehyde and with orcinol and in the intense discoloration on heating even with dilute acid. The compound, on elementary analysis, was given the formula $C_{11}H_{21}O_9N$; it does not contain acetyl groups. Recently Klenk and Faillard[26a] isolated N-acetyl neuraminic acid, $C_{12}H_{21}O_{10}N$, from bovine submaxillary gland mucoprotein. This acid exhibits the same general properties as sialic acid; unlike the methoxy derivative it has reducing power, is ninhydrin negative, and, most important, produces 2-carboxy-pyrrole on mild alkali treatment. Finally, it was shown by Klenk, Faillard, and Lampfrid[27] that the enzymatically released split

TABLE 1.

	C	H	N
N-Acetyl neuraminic acid			
(a) Calculated for $C_{12}H_{21}O_{10}N$	42.48	6.23	4.13
(b) Calculated for $C_{11}H_{19}O_9N$	42.72 (+0.6%)*	6.15 (−1.3%)	4.53 (+9.7%)
Found	42.43, 42.17 (0.6%)†	6.27, 6.32 (0.8%)	4.07, 4.11, 4.11 (1.7%)
Methoxy neuraminic acid			
(a) Calculated for $C_{11}H_{21}O_9N$	42.44	6.80	4.50
(b) Calculated for $C_{10}H_{19}O_8N$	42.70 (+0.6%)*	6.76 (−0.6%)	4.98 (+10.7%)
Found	{42.26, 42.48 / 42.51, 42.65} (0.9%)†	{6.84, 6.97 / 7.03, 7.10} (3.8%)	{4.31, 4.43 / 4.51, 4.73} (9.7%)

* Denotes increase (+) or decrease (−) of b with respect to a.
† Denotes difference between extreme values.

product from UM on purification is identical with N-acetyl neuraminic acid. In our opinion the relationship between sialic acid, N-acetyl neuraminic acid, and 2-carboxy-pyrrole on the one hand and between sialic acid and the methoxy derivative of neuraminic acid on the other hand is best expressed by the structures IV-IX.

This scheme requires a comment. Whereas Klenk and associates[26a, 27, 28] consider N-acetyl neuraminic acid and the methoxy derivative of neuraminic acid to have the formulae $C_{12}H_{21}O_{10}N$ and $C_{11}H_{21}O_9N$, respectively, we believe that their analytical data are not at variance with the formulae $C_{11}H_{19}O_9N$ and $C_{10}H_{19}O_8N$, respectively, as assigned to the two compounds in V and IX. In Table 1 the calculated values for the elements according to the composition assigned to them by Klenk and assigned to them in V and IX are compared with the data published by Klenk and associates.[26a, 26b, 27, 28] As may be seen from the figures for methoxy neuraminic acid, the differences between the C, H, and N values for the two assumed formulae are not larger than the differences between two individual analyses

of these elements, i.e., they fall within the experimental error of the method. The same holds for the C and H values of N-acetyl neuraminic acid. The analytical figures for N of N-acetyl neuraminic acid, taken at their face value, seem to fit Klenk's formula better than V. An examination, however, of the experimental details provided by Klenk shows that due to the very small amount of material (2.04-2.40 mg.) available for the Kjeldahl procedure a difference of 0.04 ml. (one drop) of N/100 HCl which is within the error of the method introduces an experiment error of $\pm$ 7 p.c. under these conditions. We conclude from the above analysis of Klenk's figures that they do not allow a differentiation between the formulae as proposed by him and as suggested in V and IX, respectively; they are consistent with our interpretation.

From Blix's data[2] it seems very probable that sialic acid from BSM is diacetyl neuraminic acid (IV) and it may be useful to restrict this name to the diacetyl derivative* of neuraminic acid. The details of Blix's latest procedure have not yet been published; there remains the uncertainty that different preparations of bovine sialic acid though exhibiting the same X-ray diagrams are not consistent enough in their elementary analysis to establish definitely the composition of BSM sialic acid.[2] Should the presence of an O-acetyl group in this compound be confirmed, then N-acetyl neuraminic acid (V) as obtained from BSM by Klenk[26a] is an artifact derived from sialic acid (IV) by loss of the O-acetyl group during purification. The same may be true for the split product released by the viral enzyme from UM.[27] Blix and associates[2] have prepared from ovomucin "ovine sialic acid" of the composition $C_{11}H_{19}O_9N$ containing an N-acetyl group only and almost certainly identical with N-acetyl neuraminic acid. "Porcine sialic acid" has no acetyl group but instead an N-glycolyl group.[2]

It would be most desirable to establish firmly whether or not the N-substituted neuraminic acids of bovine and porcine submaxillary gland mucins, of UM, and of ovomucin have an O-acetyl group in addition to the N-acyl group, because this question has wider implications. N-Acetyl neuraminic acid in solution is unstable; it decomposes under discoloration within days even at 1° C.[27] If, as Klenk[26a, 27] assumes, N-acetyl neuraminic acid is present as such in BSM and UM, it must be stabilized by its linkage to the adjacent unit within the mucoprotein molecule; for an 1 p.c. dispersion of ovomucin containing 0.04 p.c. lysozyme (final concentration) may be kept at 0° C. for months without losing its biological activity or changing its snow-white colour. Stabilization of V other than by O-acetyl (IV)

*To Blix[1] goes the credit of having first isolated this compound from BSM. It seems, however, inadvisable to denote with the same name compounds of different chemical composition though they may be closely related to or derived from sialic acid.

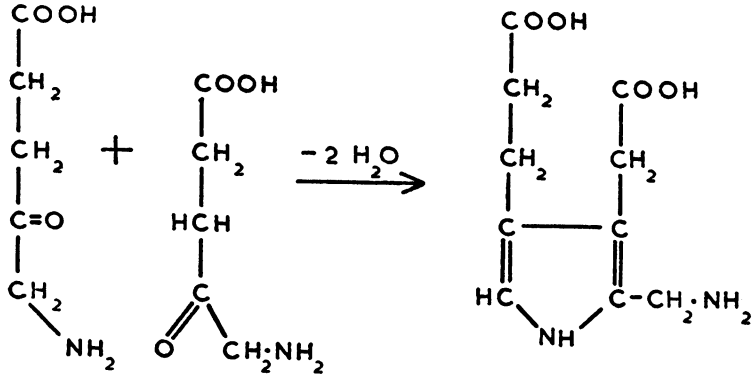
would result from the engagement of its keto group in a glycosidic linkage in analogy to IX. An ordinary O-glycosidic linkage may be excluded from the facts that both the native mucoprotein and N-acetyl neuraminic acid form 2-carboxy-pyrrole on alkali treatment,[14, 26a] whereas the acetal or O-glycoside methoxy neuraminic acid does not (10N NaOH, 2 hr., 100° C.).[26a] An N-glycosidic linkage mediated by the amino group of an adjacent amino acid or amino sugar would stabilize V efficiently and would explain the release of sialic acid from BSM by the simple expedient of heating its solution at neutrality to 110-115° C.;[1] for many synthetic N-glycosides are known to hydrolyse under very mild conditions, some of them even at pH 7.0.[13, 29, 33] Such a linkage would in no way hinder the formation of 2-carboxy-pyrrole from mucoproteins on alkali treatment; moreover, its cleavage by the action of RDE, involving the loss of a $-COO^-$ group and the gain of a $-NH_3^+$ group, would account for the considerable reduction in electrophoretic mobility of BSM and UM after such treatment.[7, 34] In this context it is of interest that Inouye and Onodera[23] and Dische and Jacubeit[8] have isolated from glycoproteins compounds consisting of a mono- or disaccharide in N-glycosidic linkage with the amino group of an amino acid.

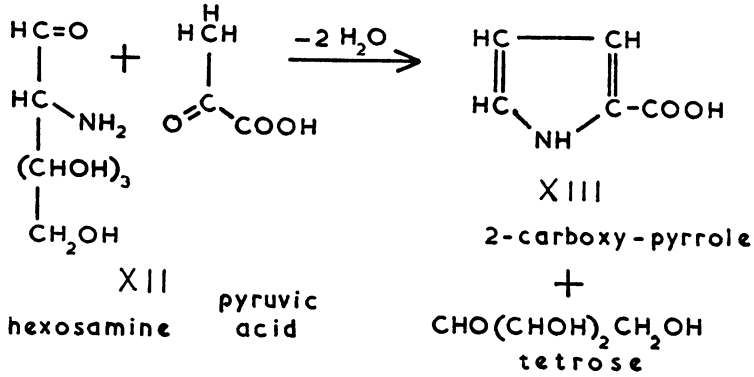
Though the scheme presented seemed to be consistent with the data provided by the Swedish, German, and Australian laboratories, it was desirable to obtain further experimental evidence for its validity. The structure assigned to neuraminic acid (VI) may be visualized as an aldol type of condensation product of 2-amino-2-deoxyhexose with pyruvic acid, a type of linkage rendering recovery of the amino sugar by acid treatment impracticable and yet allowing the masked hexosamine to play its full part in the chromogen formation in the direct and indirect Ehrlich reactions, as was indeed observed.[3, 12, 25] The tendency of pyruvic acid to condense with aldehydes has long been known; thus the reaction

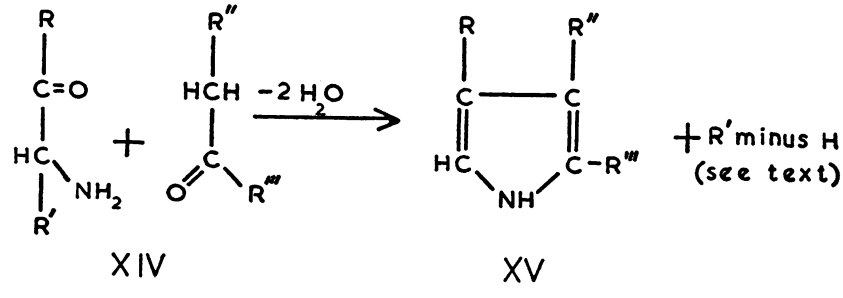
$$C_6H_5 \cdot CHO + CH_3 \cdot CO \cdot COOH \xrightarrow{-H_2O} C_6H_5 \cdot CH : CH \cdot CO \cdot COOH$$

proceeds at 5° C. when carried out in the presence of NaOH (Erlenmeyer[10]). On this reasoning it was expected that under alkaline conditions pyruvic acid would condense with hexosamine to form VI, a reaction accompanied by closure of the ring, loss of 2 moles water, rearrangement of the double bonds and elimination of a tetrose (VI → VII). This was verified experimentally.[18] When pyruvic acid (0.1 N solution) was allowed to react with D-glucosamine in the molecular ratio 1 : 3 at 100° for several hours under controlled conditions of alkalinity, 2-carboxy-pyrrole could be extracted from the reaction mixture in a yield of about 20 p.c.; the compound was obtained in crystalline form and identified so far by its melting

point, mixed melting point, U-V absorption spectrum, and chromatographic behaviour.[19] D-Galactosamine was found to be as suitable a partner in the condensation reaction as D-glucosamine, both yielding 2-carboxy-pyrrole.[19] The condensation of hexosamine and pyruvic acid to the pyrrole







compound may be regarded as an analogue to the Knorr type condensation of 2 mols of δ-amino-laevulinic acid to the pyrrole derivative porphobilinogen (X-XIII), demonstrated by Shemin and Russell[35] and by Neuberger and Scott.[31] In both cases the same reactive groups are involved in the condensation (XIV and XV). The main difference is that in δ-aminolaevulinic acid R′ = H and on condensation 2 mols H_2O are formed concomitant with pyrrole synthesis; in hexosamine R′ = $-CHOH \cdot (CHOH)_2 \cdot CH_2OH$ and upon condensation with pyruvic acid fragmentation of the hexosamine structure by reverse aldolization takes place resulting in the production of 2-carboxy-pyrrole, 2 mols H_2O and a tetrose; the latter is further degraded under the prevailing alkaline conditions.

Neuraminic acid (VI), being an α-keto acid, is readily decarboxylated by mineral acid to form the next lower aldehyde. This aldehyde may be regarded as a substituted 2-deoxyhexose and it is due to this structural feature that sialic acid gives a very similar colour reaction (violet colour with maximum absorption at 530 mμ) with Dische's diphenylamine reagent as do 2-deoxyhexoses[22a] and glycals (red colour with maximum absorption 515 mμ). Quite probably the same structural feature of 2-deoxy-sugar is responsible for the decomposition with discoloration of sialic acid and N-acetyl neuraminic acid on heating with dilute mineral acid, 2-deoxy-glucose,[36a] and 2-deoxy-galactose* exhibiting the same property. It is thus evident that all properties described for sialic acid, N-acetyl neuraminic acid and the split product are consistent with structures IV and V; the exact position of the O-acetyl group in IV remains to be determined.

From the previous discussion it is obvious that the direct Ehrlich reaction as given by BSM, UM and ovomucin is due to the presence in them of N-acetyl neuraminic acid or of diacetyl neuraminic acid (sialic acid). There is no indication that these or similar mucoproteins contain any other component but neuraminic acid reacting after rearrangement directly, i.e. without alkali pretreatment, with Ehrlich reagent to give a stable colour with absorption maxium at 565 mμ under standard conditions as defined by Werner and Odin.[37] This statement is based on two series of observations: (1) The following substances failed to give a positive direct Ehrlich reaction as defined above: Hyaluronic acid, chondroitin sulfuric acid, heparin, carbohydrate-free proteins, tryptophan.[17] 2. The determination of "sialic acid" in a variety of mucoproteins by independent methods (diphenylamine reaction, tryptophan-perchloric acid reaction) gave values varying by not more than 10 p.c. from those obtained by the method based on the direct Ehrlich reaction.[37] It seems therefore legitimate to apply the

* Dr. E. L. Hess, Chicago, personal communication.

direct Ehrlich reaction as an approximate measure of the neuraminic acid content of a mucoprotein though a check by an independent method may be desirable. The value obtained is approximate only since it is customary to take Blix's sialic acid preparation from BSM as standard and we cannot be absolutely sure that the various neuraminic acid derivatives (N-acetyl neuraminic acid, N-glycolyl neuraminic acid, and possibly others) give molecule for molecule under standard conditions the same colour intensity as sialic acid. With this reservation Table 2 summarizes the amounts in some mucoproteins of direct Ehrlich-reacting neuraminic acid derivatives expressed as sialic acid.

TABLE 2. "SIALIC ACID" CONTENT* OF VARIOUS MUCOPROTEINS

Mucoprotein	*Sialic acid p.c.*	*References*
Bovine submax. gland mucoprotein	16.8	37
Bovine serum albumin	1.7†	21
Acid glycoprotein of human plasma	10.1	37
Seromucoid (Rimington)	8.5	37
α_2-Globulin (Cohn's fraction IV-6-2)	5.7	37
Human urine inhibitory mucoprotein	9.2	32
Human meconium	8.7†	21
Blood-group active mucoids from ovarian cyst fluids	1.1-8.2	11
Thyroglobulin	2.7†	21
Ovomucin	5.8	37
Ovomucoid	2.0†	21

* See text.
† Standard = homogeneous bovine submax. gland mucoprotein assuming a content of 16.8 p.c. sialic acid.

In contrast to the direct Ehrlich reaction a positive Ehrlich reaction of a mucoprotein after mild alkali treatment (0.05 N Na_2CO_3, 15 minutes, 100° C.), first observed by Morgan and King,[30] does not lend itself to a ready interpretation. This indirect Ehrlich reaction is a complex reaction involving neuraminic acid, N-acetylglucosamine, N-acetylgalactosamine, N-glycosyl amino-acids and possibly other substances. The mechanism of this reaction is not fully understood due to the variety of reactions sugars are undergoing under the impact of alkali (enolization, condensation, fragmentation, recombination, disproportionation, etc.[88]). Several chromogens are formed in the reaction. Stanley,[36] participating in our earlier work, has separated by dialysis material liberated by 0.05 N Na_2CO_3 (20 minutes, 100° C.) from ovomucin and demonstrated chromatographically the

presence of four different substances reacting after this treatment with Ehrlich reagent; in addition to these chromogens, unchanged free N-acetylglucosamine was found to be present. We have submitted BSM to the same mild alkali treatment for 15 minutes and detected in the concentrated dialysate three different chromogens by chromatography (solvent: n-butanol / pyridine / water; R_F values 0.15, 0.86, 0.90) reacting with Ehrlich reagent. After acid hydrolysis of the dialysate an amino compound was isolated by adsorption to and elution from Dowex 50 according to Boas[4] and identified as galactosamine, the predominant amino sugar of BSM,[20] by oxidation with ninhydrin to lyxose. In this way 4 p.c. of the hexosamine known to be present in BSM was recovered unchanged. In view of these findings it seems rather arbitrary to compare the colour produced in the Ehrlich reaction of a mucoprotein after mild alkali treatment with an N-acetylglucosamine standard; until more is known about the chemistry and origin of the various chromogens formed and their colour intensity in the Ehrlich reaction compared with that given by N-acetylglucosamine, little information is conveyed by determining quantitatively the "N-acetylhexosamine colour"[11] of a mucoprotein.

From the evidence discussed in this paper it would appear that the biological activity of a group of mucoproteins, as displayed by their inhibitory effect upon haemagglutination by heated influenza virus, resides in the N-acetyl neuraminic acid component of these conjugated proteins; this has been established for BSM, UM, and ovomucin. Whether or not one or more of them contain in addition an O-acetyl group has yet to be proven; for BSM the presence of neuraminic acid in its diacetyl form seems extremely probable. Neuraminic acid has been shown with a high degree of probability to be an aldol type of condensation product of hexosamine with pyruvic acid. The release of N-acetyl neuraminic acid, catalysed by the influenza virus enzyme and by a soluble enzyme from vibrio cholerae, irreversibly inactivates the mucoprotein. N-Acetyl neuraminic acid represents, therefore, the functional group of the virus haemagglutinin inhibitory mucoproteins, either on its own or as the specific component of a terminal unit.

Klenk, Faillard, and Lempfrid[27] have confirmed our previous observation[28] that paper chromatography of the split product reveals the presence of several amino acids. However, the authors apparently have not yet tested the controls for these ninhydrin reacting substances; the matter is still under investigation. In our experience BSM, UM, and ovomucin release, for some unknown reason, continuously minute amounts of amino acids; the chromatographic analysis of the split product has, therefore, carefully to be checked against the chromatograms obtained from appropriate con-

trols. If this is done, possibly one or two amino acids remain, and their release may be associated with the virus-mucoprotein interaction.[12]

It is to be hoped that continued research will consolidate our knowledge about the chemistry of the various neuraminic acid derivatives present in mucoproteins and lead to the chemical and enzymatic synthesis of N-acetyl neuraminic acid. From such studies there might eventually emerge a clearer concept of the function of neuraminic acid which seems to represent a unique example of a fusion product between a static and a dynamic sugar derivative.

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