by furfural alone, it is probably the product of condensation of the —CHO group of furfural (HMF in hexoses) with the sugar molecule.

## SUMMARY

1. The conversion of several sugars to furan derivatives at 60° in 28·3 N-sulphuric acid was followed spectroscopically. Variations in the apparent yields and in the shapes of the absorption spectra were attributed to two other absorbing compounds, which were formed at the expense of the furan derivatives in amounts dependent on the configuration of the sugars.

2. Another by-product was formed by three of the sugars when 37·1 N-sulphuric acid was used. Its absorption exceeded that of the furfural, and caused a complete change in the appearance of the spectra.

3. The colours formed when sugars were heated at 100° in 28·3 N-sulphuric acid were found to be due to three main absorption bands. The nature of the absorbing substances is discussed.

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# Studies on Influenza Virus Receptor-Substance and Receptor-Substance Analogues

## I. PREPARATION AND PROPERTIES OF A HOMOGENEOUS MUCOID FROM THE SALIVARY GLAND OF SHEEP

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Agglutination of red cells by influenza virus, first described by Hirst (1942), may be inhibited by specific antibody, by non-specific serum inhibitors (Burnet & McCrea, 1946; McCrea, 1946) and by a wide range of mucoid substances. Relatively weak inhibition of viral haemagglutination by apple pectin was described by Green & Woolley in 1947, but this apparently non-specific effect has not been confirmed by other workers. Inhibition by serum mucoid and crude human 'mucins' (Burnet, McCrea & Anderson, 1947) appears, however, to be a more specific reaction, since the inhibitory power of such substances is destroyed on incubation with living influenza virus or the bacterial receptor-destroying enzymes (Burnet, McCrea & Stone, 1946; Burnet & Stone, 1947) in the same highly characteristic fashion as are the 'true' receptors on the erythrocyte surface.

Although purified human serum mucoid (McCrea, 1948*a*; Hirst, 1949) and ovarian-cyst mucopolysaccharide (McCrea, 1948*b*) show high activity in inhibiting haemagglutination, it has not yet been definitely established that such activity is an integral part of the mucoid molecule. Other mucoid substances such as ovomucin (Lanni & Beard, 1948; Gottschalk & Lind, 1949), human cervical mucin (Burnet, 1948*a*) and the allantoic-fluid inhibitor (Svedmyr, 1948), which show high antihaemagglutinin activity, have generally been used in a relatively crude form. Until a virus-inhibitory mucoid has been obtained in a homogeneous state,

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therefore, it might well be argued that the anti-haemagglutinin activity of such substances is due to some contaminating factor.

Anderson (1950) observed that a thermostable inhibitor of influenza virus haemagglutinin was present in the salivary glands of ferrets and sheep, and an extremely active electrophoretically homogeneous mucoprotein can readily be isolated from the latter source (McCrea, 1951). This mucoprotein has now been studied in greater detail and the present paper describes the preparation of a substance which moves as a single, sharply defined component on both electrophoresis and ultracentrifugation. Further, the mucoid shows exceptionally high anti-haemagglutinin activity against influenza B virus and moderate activity against certain influenza A strains. An essentially homogeneous mucoprotein recently isolated from urine (Perlmann, Tamm & Horsfall, 1952) shows activity of closely similar order.

## METHODS

### *Analytical methods*

Nitrogen was determined by the micro-Kjeldahl method in a Markham still (Markham, 1942), phosphorus by the method of Briggs (1922), reducing sugar by the method of Somogyi (1937), and methyl pentose (fucose) according to the method of Dische & Shettles (1948) in a Uvispek spectrophotometer. *N*-Acetylhexosamine was determined by a modification of the colorimetric method of Morgan & Elson (1934), hexosamine by a modification of the method of Elson & Morgan (1933) and also by the method of Dische & Borenfreund (1950). In spite of careful purification of all the reagents used in the latter procedure, including repeated crystallization and sublimation of the indole, high absorption occurred in the blank solutions; in our hands this method was less sensitive and more cumbersome than the Elson & Morgan (1933) procedure. Derivatives of the hexosamine component obtained on acid hydrolysis were prepared by the methods of Jolles & Morgan (1940) and Annison, James & Morgan (1951).

Paper chromatography was carried out for amino acids as described by Consden, Gordon & Martin (1944) and for sugars and amino sugars as described by Partridge (1948). Whatman no. 4 paper was used throughout.

### *Titration of viral antihaemagglutinin*

The antihaemagglutinin activity of the preparations was determined as described by Anderson (1948). A series of double dilutions of mucoid was made in 0·2 ml. of physiological saline, an equal volume of saline containing 5 agglutinating units of influenza virus was added to each tube, and, after standing at 4° for 1 hr., a further volume of 1% (v/v) sensitive fowl red cells (Anderson, 1948) or pigeon cells was added. The inhibition titre was read from the pattern of deposited cells after standing for 1 hr. at 4°. The 'indicator' forms of influenza A and B virus were prepared by heating allantoic fluid under the conditions described by Stone (1949*a*). The virus strains, which were kindly provided by Dr A. Isaacs, of the World Influenza Centre, Mill Hill, London, were reference strains cultivated by the standard suballantoic method. Purified receptor-destroying enzyme of *Vibrio cholerae* (Burnet & Stone, 1947; Ada & French, 1950) was obtained through the courtesy of Sir MacFarlane Burnet.

## EXPERIMENTAL

### *Isolation of the purified mucoid*

*Extraction of salivary glands.* The material used in the present series of experiments was obtained from sheep which had been inoculated 4 days previously with vaccinia virus. Immediately after the sheep had been killed, the submandibular glands were excised and dehydrated for 2–3 months in several changes of absolute ethanol. The glands were then minced finely, air-dried for several hours at room temperature, and extracted with water in a Waring Blendor, allowing approximately 300 ml. of water to each 100 g. glands. The suspension was brought to 80° for 2 min., cooled to room temperature, and shaken for 6 hr. After standing overnight at 4° in the presence of $CHCl_3$, the insoluble debris was removed by filtration and finally by centrifugation at 8000 rev./min. in a Servall angle centrifuge (model XL), and the supernatant fluid dried from the frozen state. Only a small amount of active material could be obtained on further extraction of the gland debris. The average yield of crude mucoid was 30 g./kg. of dry glands.

In earlier experiments (McCrea, 1948*b*), highly active virus-inhibitory mucoid was prepared by extracting freshly excised submandibular glands of normal sheep without preliminary dehydration in ethanol. Such material was closely similar in activity and chemical composition to that described in the present paper, but was markedly polydisperse in the ultracentrifuge. Some preparations were made by precipitation at 4° with acetic acid; a firm clot closely resembling hyaluronic acid was formed, but this procedure yielded material of low activity and destroyed physical properties such as the viscosity and 'spinnbarkeit' characteristic of crude salivary mucoid obtained by other methods. Significant loss of activity also occurred on treatment with trichloroacetic acid in the cold.

*Fractionation with ethanol.* In a typical experiment, a 10% (w/v) aqueous solution of crude mucoid was cooled to 4° and ethanol run in slowly with constant stirring in the presence of potassium acetate. The fraction separating between 40 and 66% (v/v) ethanol concentration, which contained practically all the activity, was collected on the centrifuge, dialysed against distilled water at 4°, and dried from the frozen state; yield 47% of the original weight (fraction *A*). A second fraction (fraction *B*) collected between 75 and 80% (v/v) ethanol concentration represented 17% of the original material. Fraction *A* dissolved slowly to form a turbid viscous solution; fraction *B* dissolved almost instantaneously giving a water-clear solution.

*Treatment with chloroform-butanol mixture.* In one experiment, 3·4 g. of fraction *A* were dissolved in 320 ml. of Palitzsch's borate buffer, pH 6·8, 140 ml. of 9:1 (v/v) chloroform-butanol mixture was added, and the emulsion stirred vigorously for 1 hr. The resulting gel was separated by centrifugation, and the procedure repeated until no

further insoluble material formed. After dialysis, the supernatant fluid was dried from the frozen state, yielding 1·3 g. of snow-white powder (fraction *C*I). No decrease in activity occurred.

In another experiment 13·5 g. of fraction *A* were treated similarly with the exception that borate buffer was replaced by 0·2M-acetate buffer, pH 4·7. As will be described in a later section, the most completely homogeneous material (fraction *C*II) was obtained in this way.

### *Properties of the purified mucoid (fractions C I and C II)*

*Physical properties.* Fractions *C*I and *C*II dissolved rapidly in water giving water-clear non-viscous solutions. The mean specific rotation was $[\alpha]_{5461} + 18°$ in water at room temperature. The ultraviolet-absorption spectrum examined in a Uvispek spectrophotometer showed a well defined absorption peak at 255–260 mμ. (Fig. 1), presumably due to the presence of nucleoprotein.

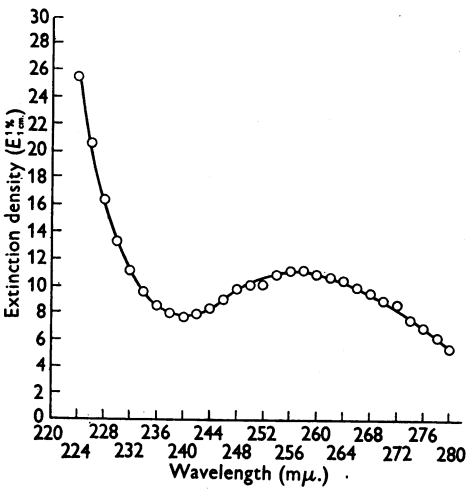


Fig. 1. Ultraviolet-absorption spectrum of fraction *C*I; 0·05% (w/v) solution in 0·85% (w/v) NaCl.

The physical homogeneity of the preparations was examined in the ultracentrifuge and electrophoresis apparatus; details of these studies are given in the addendum by Mr E. A. Caspary. In summary, all preparations migrated as one sharply defined component on electrophoresis, fraction *C*II remained as a single component even after prolonged centrifugation at full speed (270 000 *g*) in the ultracentrifuge, whilst fraction *C*I showed two closely related peaks after prolonged centrifugation.

*Chemical properties.* No precipitate occurred when equal volumes of the following reagents were added to a 2% (w/v) aqueous solution of fractions *C*I or *C*II: trichloroacetic acid (20%), sulphosalicylic acid (10%), phosphotungstic acid (2%), tannic acid (5%), picric acid (5%), basic lead acetate (2%) (all w/v). There was no precipitation on boiling. The ninhydrin and biuret tests were strongly positive, the Millon test weakly positive; some preparations gave a weak Molisch reaction. The Dische (1947) test for hexuronic acid was negative.

Analytical figures for a typical preparation (fraction *C*I) were: N, 10·1%; P, 0·4%; S, 1·4%*; ash 1·4%. The phosphorus content varied considerably in various preparations (one sample contained no detectable phosphorus), but the other figures did not change significantly. The maximum amount of reducing sugar, calculated as glucose and

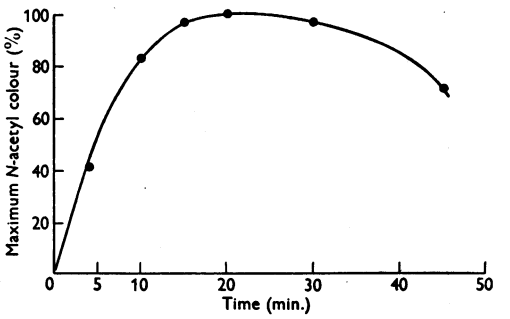


Fig. 2. Development of *N*-acetylhexosamine colour reaction on heating purified mucoid *C*I with 0·05N-$Na_2CO_3$ at 100°.

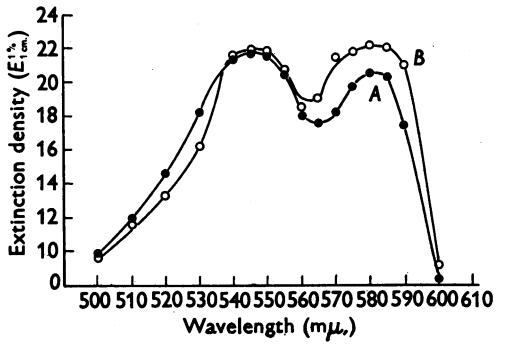


Fig. 3. Absorption spectra of the colour produced by purified mucoid, fraction *C*I (*A*), and pure *N*-acetylglucosamine (*B*), after heating with 0·05N-$Na_2CO_3$ and treatment with Erhlich's reagent.

released after 48 hr. hydrolysis in 0·5N-hydrochloric acid, was 13·6%. The mucoid gives the colour reaction for *N*-acetylhexosamine on heating with 0·05N-sodium carbonate and treatment with Ehrlich's reagent (Morgan & Elson, 1934), the maximum colour obtained being equivalent to 4·1%, after heating at 100° for 20 min. (Fig. 2). The absorption spectrum of the colour obtained in the *N*-acetylhexosamine test, which shows two well

* S determined by Weiler and Strauss, Oxford.

defined maxima, corresponded closely to that of a known specimen of *N*-acetylglucosamine (Fig. 3) (Aminoff, Morgan & Watkins, 1952).

After hydrolysis in 5N- or 0·5N-hydrochloric acid, the maximum hexosamine, calculated as glucosamine base, was 12·4% (Elson & Morgan, 1933), or 16·7% by the method of Dische & Borenfreund (1950). About 1% methyl pentose (fucose), determined by the method of Dische & Shettles (1948), was present in all preparations.

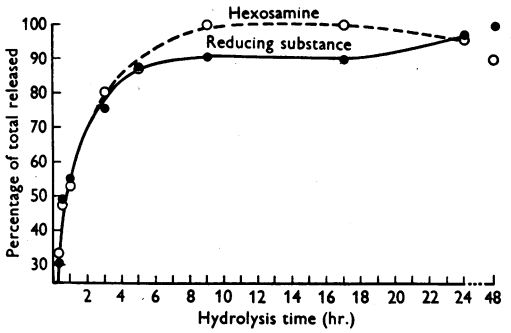


Fig. 4. Release of reducing substances (calculated as glucose) and hexosamine (as glucosamine base) on hydrolysis of fraction *C*I with 0·5N-HCl at 100°.

*Hydrolysis curve.* Release of reducing substances and hexosamine was followed by sealing samples of a 1% (w/v) aqueous solution of fraction *C*I with an equal volume of N-hydrochloric acid in small ampoules, and immersing in a water bath at 100°. The ampoules were removed at the intervals shown on the curve (Fig. 4), the contents neutralized, and determinations carried out in triplicate by the methods described. The curve shows that the maximum amount of hexosamine (12·4%) was released after hydrolysis for 9 hr., and decreased slightly after 17 hr.; at 9 hr. 90% of the total reducing substances have been released, but the maximum (13·6%) was not reached until 48 hr.

### *Isolation of hexosamine derivatives*

Attempts were made to isolate crystalline derivatives of the hexosamine component released on hydrolysis of purified mucoid with acid. With this mucoid, however, it appears that it is much more difficult to obtain such compounds than had been found with blood-group A substance (Annison *et al.* 1951).

In one experiment, fraction *C*II (500 mg.) was hydrolysed at 100° for 16 hr. with 0·5N-HCl under a stream of $N_2$. The hydrolysate was taken to dryness *in vacuo* and dissolved in 10 ml. of 0·4M-borate buffer at pH 9, according to the procedure of Annison *et al.* (1951). The solution was cooled to 0°, the pH adjusted to 9·5 by the addition of NaOH, and 3 ml. of 1:2:4-fluorodinitrobenzene added in ethanol solution. After standing for 3 days at 0°, the dark-orange solution was evaporated to dryness *in vacuo* at room temperature. A few drops of amyl alcohol:chloroform mixture were added and the moistened powder transferred to the top of a 50 g. kieselguhr column, and developed in 30% (v/v) amyl alcohol:chloroform mixture at 2°. After discarding some fast-moving impurity, a slow-moving orange band was collected. This was concentrated to dryness *in vacuo* at 40° and the material was run twice more on kieselguhr columns in the same way. The product, taken to dryness *in vacuo*, was crystallized twice from 80% (v/v) acetone-water mixture, and once from 0·5 ml. of acetone at −10°. A small yield (3 mg.) of orange crystals was obtained, m.p. (uncorrected) 160–162°. An authentic sample of *N*-2:4-dinitrophenyl galactosamine melted at 170–172°. The experiment was repeated twice with similar results.

An attempt was also made to obtain a 2-hydroxynaphthaldehyde derivative of the hexosamine (Jolles & Morgan, 1940). Fraction *C*I (400 mg.) was hydrolysed as before, taken to dryness *in vacuo* over NaOH, and treated with excess (400 mg.) 2-hydroxynaphthaldehyde. The crude Schiff's base was taken up in a minimum of warm methanol, treated with acetone, and stood for several days at −10°. No crystals separated, however, even after 'seeding' with 2-hydroxynaphthylidenegalactosamine. Satisfactory yields of the crystalline derivatives of glucosamine and galactosamine were obtained when the pure hexosamines were treated in the same way.

### *Chromatographic examination of the hydrolysed mucoid*

Portions of the hydrolysed mucoid were examined for sugars, hexosamines, and amino acids by the technique of paper chromatography.

*Hexosamine.* One cherry-red spot was obtained when the hydrolysed material was run in collidine and sprayed with the hexosamine reagents (Partridge, 1948); the spot showed the same $R_F$ value (0·28) as 'known' galactosamine run on the same paper strip. A further spot appeared ahead of the 'unknown' spot if glucosamine was added to the hydrolysate, but not if galactosamine was added (cf. Aminoff & Morgan, 1948). This effect was best shown if the chromatograms were run for 48 hr. at 4°.

*Sugars.* Hydrolysed material (0·5N or 5N-HCl) was run in collidine, butanol:acetic acid and phenol:ammonia, and sprayed with the silver nitrate (Partridge, 1948) and aniline hydrogen phthalate (Partridge, 1949) reagents. Apart from the hexosamine spot, however, no clearly defined spots were obtained.

*Amino acids.* Material hydrolysed with 6N-HCl was examined by two-dimensional chromatography, the solvents being collidine and phenol (Consden *et al.* 1944). The following amino acids, and chondrosamine, were identified: aspartic acid, glutamic acid, glycine, arginine, serine, alanine, threonine, valine, leucine and isoleucine.

### *Virus antihaemagglutinin activity*

*Inhibition spectrum.* A 1% (w/v) aqueous solution of fraction *C*I was titrated against a number of active and indicator influenza virus strains using chicken red cells. The results are shown in Table 1.

It will be seen that this preparation shows extremely well the Francis phenomenon (Francis, 1947), namely that indicator influenza B virus, strain LEE, heated to 56° for 30 min., is very much more sensitive to inhibition than is the active unheated form: in the present instance there is an almost 1000-fold increase in titre against the indicator virus. With the influenza A virus strains, PR 8 shows an approximately tenfold increase in sensitivity to inhibition after conversion to the indicator form, whilst strain MEL, as was previously observed by Stone (1949*b*) is inhibited to only a slight extent by sheep salivary mucoid. Swine influenza (Shope strain 15) is also inhibited to relatively low titre. Fraction *B* (precipitated between 75 and 80 % ethanol concentration) inhibited heated LEE to a titre of 20 000. Fraction *C* II was not examined against all virus strains, but showed activity of the same order as fraction *C* I against LEE, H. LEE, PR 8, and H.PR 8.

Table 1. *Antihaemagglutinin activity of purified mucoid, fraction C I, in 1 % (w/v) aqueous solution*

| Virus strain | Titre* | Virus strain | Titre* |
|---|---|---|---|
| LEE, active | 4 800 | MEL, active | 10 |
| LEE, indicator | 3 200 000 | MEL, indicator | 150 |
| PR 8, active | 400 | SW, active | 180 |
| PR 8, indicator | 5 600 | SW, indicator | 800 |

* Reciprocal of dilution giving 50 % neutralization of virus.

### *Effect of various treatments*

*Treatment with liquid phenol.* Crude mucoid (300 mg.) was taken up in 13 ml. of 90 % (w/v) phenol (Morgan & King, 1943). The bulk of the material dissolved rapidly, and after stirring for 6 hr. only 6·2 mg. of insoluble material separated on centrifugation. A 1:1 (v/v) mixture of ethanol and phenol was cautiously added to the supernatant fluid; no precipitate formed at 50 % but a heavy precipitate separated between 55 and 75 % (v/v) ethanol concentration. The precipitate was collected by centrifugation, washed free from phenol with ethanol, taken up in water, and freeze-dried; 25·9 mg. of grey-white material was obtained. The material remaining in solution at 75 % ethanol concentration was dialysed free from phenol and freeze-dried.

The activity of the fraction precipitated with 75 % ethanol was reduced by approximately 80 % as compared with the starting material which, in 1 % (w/v) aqueous solution, inhibited H. LEE to a titre of $1{\cdot}2 \times 10^6$. The activity of the phenol-insoluble material and the fraction soluble in 75 % ethanol was reduced by approximately 95 %. Inactivation to a similar extent occurred if crude mucoid was dissolved in 90 % phenol, stirred for several hours, dialysed free from phenol and dried from the frozen state.

*Action of trypsin.* The virus-inhibitory activity of the purified mucoid is destroyed extremely rapidly on incubation with dilute trypsin.

In one experiment purified mucoid was incubated at 37° with crystalline trypsin (final concentration 1 μg./ml.) in borate buffer pH 7·5. Samples were removed at intervals, heated to 100° to inactivate the enzyme, and titrated in the usual manner against indicator LEE virus. Control tubes without enzyme and with heat-inactivated enzyme were included.

After incubation for 15 min. the titre of the enzyme-treated material was reduced to 17 % of the control titre, and after 30 min. no inhibitory activity could be detected. Loss of inhibitory activity was most rapid at the beginning of incubation, since a sample removed at zero time (immediately after adding the enzyme solution) showed only 33 % of the activity of the control. Experiments of this type will be described in detail elsewhere.

*Action of metaperiodate.* Equal volumes of potassium metaperiodate diluted in series from 0·01 to 0·001 M were added to samples of purified mucoid dissolved in 0·2 M-acetate buffer, pH 5·5 (Walpole, 1914). After standing for 1 hr. at 20° the contents of the tubes were titrated in 0·1 M-glycerol saline against indicator LEE virus.

Even with the highest dilution of metaperiodate (final concentration 0·00033 M), the titre of the mucoid was reduced to 2 % of the control value.

*Action of receptor-destroying enzymes.* Inactivation of partially purified sheep salivary mucoid by the receptor-destroying enzyme (RDE) of *V. cholerae* and active influenza virus was studied in detail by Stone (1949*b*). The mucoid obtained in the present investigation is also rapidly inactivated by RDE, as may be illustrated by an experiment in which highly purified *V. cholerae* RDE (0·001 % w/v in Walpole's 0·2 M-acetate buffer, pH 6·2) was incubated at 37° with a solution of fraction *C* I. The titre (H. LEE) was reduced by 99 % after incubation for 1 hr.

## DISCUSSION

The homogeneity of the isolated mucoid (fraction *C* II) is well established: a single sharply defined component is revealed both on electrophoresis and after prolonged ultracentrifugation. There seems little doubt, therefore, that in this instance the virus-inhibitory factor is a component of the mucoid molecule, although it does not necessarily follow that more than a small proportion of the molecule is concerned in inhibiting haemagglutination. With purified ovomucin, for example, it has been shown that antihaemagglutinin activity is concentrated in as little as 5–10 % of the total molecule (Fazekas de St Groth & Gottschalk, 1951).

The inhibitory activity of the purified mucoid against indicator influenza B virus is at least as high as that of any mucoid virus inhibitor previously

recorded. A titre of the order of $3 \times 10^6$ obtained by serial dilution under the conditions described means that in the 'end-point' tube, in which 50% of the virus has been neutralized, the final concentration of mucoid is of the order of 0·001 $\mu$g./ml. This amount of mucoid is apparently capable of combining with several thousand virus particles, thus preventing their adsorption to the erythrocyte surface with consequent haemagglutination. Antihaemagglutinin titre against active (unheated) LEE virus is approximately one-thousandth of that against the indicator form, but even so is higher than was observed for 'specific' receptor-substance mucoproteins extracted from human erythrocytes (McCrea, 1952, 1953). The inhibitory activity of a purified mucoprotein from human urine is closely similar to that of the submandibular mucoid, but the former differs in several physical respects such as high viscosity, slow solubility in water, insolubility in 0·05M-sodium chloride, and in having a high molecular weight of the order of $7{\cdot}0 \times 10^6$ (Tamm & Horsfall, 1950, 1952).

The nature of the hexosamine component of the salivary mucoid is not definitely established; it is certainly not glucosamine, but yet does not entirely correspond to galactosamine. The $R_F$ value of the unknown hexosamine is identical with that of galactosamine as determined by paper chromatography, but this alone is not sufficient evidence for its identification. The fact that the *N*-acetylhexosamine content, as judged from the Morgan & Elson (1934) reaction, is only approximately one-third of that expected from the hexosamine colour obtained after acid hydrolysis, suggests the presence of galactosamine rather than glucosamine because the intensity of the *N*-acetylhexosamine colour of the former substance is considerably less than its hexosamine colour (Aminoff *et al.* 1952). The melting point of the crystalline *N*-2:4-dinitrophenyl hexosamine obtained was 10° and 20° lower than for authentic *N*-2:4-dinitrophenyl galactosamine and *N*-2:4-dinitrophenyl glucosamine respectively. Whilst this may be due to the fact that on hydrolysis with 0·5N-acid the hexosamine was not liberated entirely free from other degradation products, it is possible that a new hexosamine is present. The finding that the Schiff's-base derivative was considerably more soluble than the authentic glucosamine or galactosamine derivatives is perhaps evidence in the same direction. In a note published during the course of the present work, Blix, Svennerholm & Werner (1950) state that human saliva and serum mucoid contain galactosamine only. Other receptor analogues such as ovomucin and ovarian cyst polysaccharide appear to contain both glucosamine and galactosamine, as do also inhibitory mucoproteins isolated from human erythrocytes (McCrea, 1952, 1953).

Finally, the relation of the soluble mucoid 'receptor analogue' to the red-cell receptor should briefly be considered. In its sensitivity to trypsin and metaperiodate, and almost complete solubility in and inactivation by liquid phenol, sheep submandibular mucoprotein resembles the serum mucoid antihaemagglutinin (McCrea, 1948*a*, Hirst, 1949) and ovomucin (Gottschalk & Lind, 1949) rather than ovarian cyst mucoid which is phenol-insoluble and relatively resistant to the action of proteolytic enzymes. Virus-inhibitory mucoproteins isolated by various methods from human erythrocytes are phenol-soluble but resistant to trypsin (McCrea, 1952, 1953). The action of metaperiodate and proteolytic enzymes on the homogeneous virus inhibitor of human urine (Tamm & Horsfall, 1952) has not yet been described. Sheep salivary mucoid is progressively inactivated by living influenza virus (Stone, 1949*b*), by receptor-destroying enzymes, and by the periodate ion, properties which are all duplicated by the virus receptor on the intact red-cell surface. Inactivation by living virus of both the cell receptor and soluble mucoids is a specific enzymic reaction (Hirst, 1948; Burnet, 1948*b*; Stone, 1949*b*; Tamm & Horsfall, 1952), which takes place under similar conditions and with the same end result, namely loss of the capacity to adsorb further virus. It may, therefore, be concluded that similar chemical groupings exist in the erythrocyte receptor and the mucoids, and that the virus-inhibition phenomenon is in essence a form of substrate competition. The exceptionally high activity of sheep salivary mucoid may thus be due to a greater proportion, or availability, of those groupings which are enzymically modified by living virus and to which indicator virus remains firmly adsorbed. The precise nature of the chemical groupings involved is not yet known; apart from the amino acids found in all these mucoids, the only common factor appears to be hexosamine. An alkali-labile *N*-acetylhexosamine-amino-acid complex is released as the split product after incubation of ovomucin with influenza virus (Gottschalk, 1949, 1951), a finding which suggests that the hexosamine component of this receptor analogue may be involved in the enzymic action of influenza virus.

## SUMMARY

1. The isolation from the submandibular glands of sheep of a mucoprotein with high antihaemagglutinin activity against influenza B virus is described.

2. The mucoprotein was homogeneous on electrophoretic and ultracentrifugal examination. Analysis gave the following figures: N, 10·1; P, 0·4; S, 1·4 and fucose 1·1%. On acid hydrolysis 13·6% of reducing substances and 12·4% of hexosamine were released. The ultraviolet-absorption spectrum indicated the presence of nucleic acid.

3. One hexosamine was revealed by paper chromatography; the $R_F$ value was identical with that of galactosamine, but the nature of the hexosamine has not definitely been established. The hydrolysate contained ten amino acids.

4. Agglutination of red cells by indicator influenza B (LEE) virus was inhibited by the purified mucoid at a concentration of 0·001 $\mu$g./ml.

5. Antihaemagglutinin activity was lost on treating the mucoid with receptor-destroying enzymes, potassium periodate and trypsin.

6. The relation of the mucoid to red-cell virus receptors and other soluble mucoid antihaemagglutinins is discussed.

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ADDENDUM

# Physicochemical Examination of a Mucoid from the Salivary Gland of Sheep

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The sheep salivary mucoid was examined in the electrophoresis apparatus and ultracentrifuge, and in addition the diffusion constant and partial specific volume were determined.

## METHODS

*Electrophoresis.* The material was examined in a Tiselius electrophoresis apparatus at 0° using the diagonal-schlieren optical system and monochromatic light $\lambda = 546$ m$\mu$., isolated from the mercury arc by a suitable filter. Solutions were made up in suitable buffers to give concentrations of 1·5–2·0% (w/v) and dialysed for 24 hr. against buffer. These concentrations were chosen in order to detect traces of contaminating high-molecular-weight substances.

*Sedimentation.* Ultracentrifugal examination was carried out in a Svedberg oil turbine machine at 54 000 rev./min. (240 000 *g*) using a diagonal-schlieren optical system (Philpot, 1938). The variation of sedimentation constant with concentration was examined over the range 0·5–1·0% (w/v). Solutions were made up in suitable buffers with added NaCl to suppress charge effects on sedimentation.

*Diffusion.* The diffusion measurements were carried out at 25° by the Gouy interferometric method (Kegeles & Gosting, 1947) in a modification of the apparatus of Gosting, Hanson, Kegeles & Morris (1949). In order to increase the sensitivity of the method the blue line, $\lambda = 436$ m$\mu$. isolated by a suitable filter from a high pressure mercury arc, was used.

*Partial specific volume.* This was determined pyknometrically at 25°, the mucoid being dissolved in the buffer used for the sedimentation measurements.

## RESULTS

In both phosphate (pH 8, $I = 0{\cdot}2$) and acetate (pH 4, $I = 0{\cdot}1$) buffers only one component could be demonstrated after prolonged electrophoresis. At pH 8 the ascending boundary was symmetrical, the descending side showed some asymmetry. At pH 4 both boundaries were slightly skewed (Fig. 1). The spreading of both boundaries appeared similar at both pH's 4 and 8. Migration was anodic in both cases, though somewhat slower at pH 4.

In the ultracentrifuge at pH 8 only one component was demonstrable, the peak remaining relatively sharp even after 150 min. at 240 000 *g* in a 0·75% (w/v) solution, and at 0·5% (w/v) a satisfactory peak was present after 90 min. at 240 000 *g* (Fig. 1). Sedimentation constants determined at three concentrations showed a slight, apparently linear, variation of sedimentation constant with concentration (Table 1).

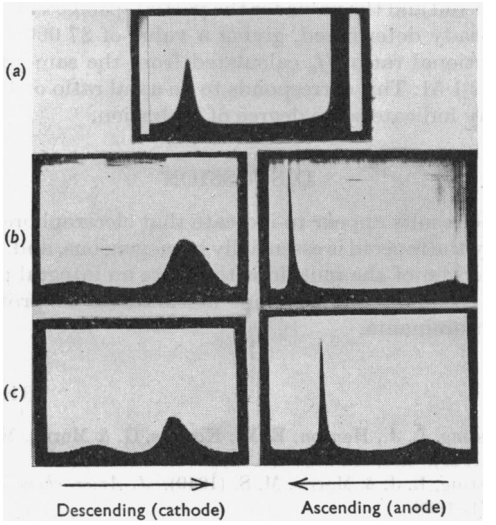


Fig. 1. Sedimentation diagram and electrophoresis patterns of sheep salivary mucoid. (*a*) Sedimentation after 105 min. at 240 000 *g*, 0·75% (w/v) in phosphate-NaCl (phosphate, pH 8, $I = 0{\cdot}2$; NaCl, 0·15 M). (*b*) Electrophoresis pattern after 37·5 mA-hr. of 1·2% (w/v) solution in acetate, pH 4, $I = 0{\cdot}1$. (*c*) Electrophoresis pattern after 27·5 mA-hr. of 1·0% (w/v) solution in phosphate, pH 8, $I = 0{\cdot}2$. Lower arrows show direction of migration in both (*b*) and (*c*).

Table 1. *Sedimentation and diffusion constants of sheep salivary gland mucoid*

(Values of sedimentation and diffusion constants corrected to water at 20° (Svedberg & Pedersen, 1940).)

| Concentration (g./100 ml.) | $S_{20} \times 10^{13}$ (corr.) | $D_{20} \times 10^{7}$ (corr.) |
|---|---|---|
| 1·00 | 1·99 | — |
| 0·75 | 2·28 | — |
| 0·50 | 2·53 | 2·27 |
| 0·25 | — | 2·31 |

The diffusion constant was measured at pH 8 and concentrations 0·5% (w/v) and 0·25% (w/v). The values of $C_t$ (the maximum downward displacement of light at the photographic plate if the light followed

geometrical optics, cf. Longsworth, 1947; Gosting & Morris, 1949) show a pronounced tendency to decrease as the fringe number $j$ increases, indicating a departure from the ideal form, defined by the probability integral. Calculations were made from the fringe $j=1$, this would approximate to $D_A$ (diffusion constant determined by height-area method).

The partial specific volume of the substance was 0·691.

The molecular weight was calculated from the usual equation (Svedberg & Pedersen, 1940), using the sedimentation and diffusion constant values obtained in a solution containing 0·5 g./100 ml. mucoid and the value for the partial specific volume, already determined, giving a value of 87 000. The frictional ratio $f/f_0$ calculated from the same data was 1·51. This corresponds to an axial ratio of 10 or may indicate some degree of hydration.

## DISCUSSION

The results appear to indicate that electrophoretically the mucoid is essentially homogeneous, and, the nitrogen of the mucoid is therefore an integral part of the molecule, and not attributable to protein contaminants.

The appearance of the ultracentrifugal boundary indicates a fairly homogeneous material, taking size and shape into consideration. Some deviation from an ideal relationship is shown in the diffusion measurements, which is not large, but suggests the presence of more than one molecular species. The molecular size and degree of asymmetry are considerably smaller than those reported by Tamm & Horsfall (1952) for a urinary mucoid of similar biological properties.

The conclusion is that the mucoid is slightly polydisperse, but the precise range and distribution of molecular size have not been calculated, since insufficient data were available.

## SUMMARY

1. The mucoid has been shown to be essentially homogeneous electrophoretically.

2. The molecular weight of the mucoid calculated from sedimentation and diffusion data is 87 000 with a frictional ratio of 1·51.

3. The mucoid is probably slightly polydisperse and not greatly asymmetrical in molecular shape.

I wish to acknowledge the technical assistance of Mr H. Murray.

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# The Proportion of Phosphatase Activity Demonstrable in Brain by Histological Techniques

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Characteristic differences have been found in brain tissue in the histological sites of hydrolysis of adenosine triphosphate (ATP), adenosine 5-monophosphate (A5MP), aneurin pyrophosphate (APP), and of glycerophosphate (GP) by the acid and alkaline phosphatases (Naidoo & Pratt, 1951, 1952). Evidence that separate enzymes were represented was provided by differences in the conditions needed and by the preferential suppression of each reaction by suitable inhibitors. The validity of these results would be more readily assessable if the proportion of the potential enzyme activity of the tissue they represented was known. This is the purpose of the present work.

Histological study of the sites of phosphatase activity is made possible by the precipitation of inorganic phosphate at the site of its formation. The hydrolysis of a suitable substrate in the presence of