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STUDIES ON INFLUENZA VIRUS RECEPTOR SUBSTANCE AND RECEPTOR-SUBSTANCE ANALOGUES

II. Isolation and Purification of a Mucoprotein Receptor Substance from Human Erythrocyte Stroma Treated with Pentane†

Agglutination of red cells by influenza virus, and the concept of "receptor" areas on the cell surface which are enzymatically modified after contact with virus, were described by Hirst in 1942.[17] Since that time much indirect evidence has been collected suggesting that virus receptors consist, at least in part, of mucoid substances; examples of this evidence are the inactivation of receptors by periodate[18] and the active competition with receptors by mucoids such as the "Francis inhibitor" of serum and ovarian cyst fluids,[8, 22, 23] ovomucin,[14, 21] and highly purified sheep salivary mucoid.[26]

Direct isolation of specific receptor-substance mucoids from the complex lipoprotein of the erythrocyte surface is obviously more difficult than the purification of receptor analogues from soluble mucoid secretions. Virus-inhibitory extracts have, however, been prepared from red cells of various species by boiling stroma in water[15] or by extracting red cells in a Waring blendor.[13] A purified preparation isolated from human red cell stroma in the first instance as an ether-soluble lipomucoid was described in 1948 by deBurgh, Yu, Howe, and Bovarnick.[5] Their most active fraction contained at least 50% reducing substances but was said to contain neither hexosamine nor phosphorus. In the same year Woolley[41] obtained a heat-labile polysaccharide substance from the supernatant fluid of hemolyzed erythrocytes which was shown to antagonize the antihemagglutinin effect of apple pectin. After exposure to influenza virus the purified erythrocyte substance was no longer capable of inhibiting pectin, and it was concluded that the former material, which itself showed detectable inhibition of PR8 virus at 0.04 μg/ml.,[42] was the substrate for enzymic attack by virus on the red cell surface. In an extensive study by Howe,[19] which appeared while the present work was in progress, physical methods were described for the isolation

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by high-speed centrifugation at controlled pH and ionic strength, of stroma fractions rich in virus inhibitor and blood-group substance. In the majority of these cases, however, detailed chemical investigations of the various preparations were not described.

Although we have found the method of deBurgh *et al.* relatively convenient to carry out in the laboratory, the yield of active material is extremely low, and, even after further purification, the most active fraction is markedly inhomogeneous in the ultracentrifuge.[25] We have also prepared an active virus-inhibitory lipomucoid by extracting dried red cells with dilute ethanol, as originally described by Hallauer[16] for the isolation of blood-group substance, but the method is cumbersome and the yield is again extremely small. It has seemed desirable, therefore, to investigate alternative methods in an attempt to improve the yield and, if possible, the homogeneity, of extracted receptor-substances.

In the present investigation, human red-cell stroma were treated with pentane in the hope that active material might be released into the solvent. Neither virus-inhibitor nor blood-group substance were, however, present in the pentane extract, but it was observed that after treatment with this solvent at room temperature for several days the stroma were converted into a stable amorphous gel. It seemed likely that stroma in this finely divided state would be suitable for further extraction, and it was subsequently found that relatively large amounts of active material passed into solution when the stroma gel was extracted with 50% ethyl alcohol. No active material was obtained if stroma were extracted without the preliminary pentane treatment.

This paper describes some of the properties of a virus-inhibitory mucoprotein obtained in relatively high yield from human group A stroma treated by the pentane-dilute alcohol process. The mucoprotein has been examined chemically and in the ultracentrifuge and electrophoresis apparatus. The inhibitory activity of the various mucoprotein fractions has been estimated against several active and indicator strains of influenza virus, and the destruction of inhibitory activity confirmed on exposing purified receptor-substance to active influenza virus or the receptor enzyme of *V. cholerae.*

MATERIALS AND METHODS

Virus. "Reference" strains of influenza A and B virus, in the form of infected allantoic fluid, were obtained through the kindness of Doctor Alick Isaacs of the World Influenza Centre, Mill Hill, London. "Indicator" forms of strains LEE, MEL, and PR8 (i.e., H.LEE, H.MEL, and H.PR8) were prepared by heating the allantoic

fluids at 37° or 55° C. under the conditions described by Stone.[38] Virus in this form is considerably more sensitive to inhibition by mucoids than active (unheated) virus.[12]

Receptor-destroyer enzyme. A purified preparation of *V. cholerae* receptor-destroying enzyme was kindly provided by Sir Macfarlane Burnet. The material had been purified as described by Ada and French.[1]

Titration of viral antihemagglutinin. The method employed throughout these studies was essentially that described by Anderson.[2] In summary, a series of double dilutions of the extracts was prepared in 0.2 ml. of normal saline; ten agglutinating units of virus in the same volume of saline were then added to each, and the mixture held at 4° C. for one hour. Finally, 0.2 ml. of a freshly prepared 1% suspension of pigeon or "sensitive" fowl erythrocytes were added, and the pattern of agglutination read 1 to 1½ hours later. In the majority of cases pigeon cells were used since they possess the added advantages of rapid settling and high stability as well as giving titres of the same order as the most sensitive fowl cells. The titres obtained with the pigeon erythrocytes from our stock did not vary significantly from bird to bird.

Particular care was taken to standardize the titrations of inhibitory activity as far as possible, since the subsequent procedures were to a large extent conditioned by them. A "standard" preparation was included in all tests, and the titrations of any one series of fractions were carried out at the same time with the same virus suspension. The latter was titrated immediately before use, diluted to give ten agglutinating doses per unit volume, and the titre of the dilute preparation checked against the freshly prepared red-cell suspension. The relatively powerful dose of ten units was used throughout because it was observed in earlier experiments with red-cell extracts that there is not necessarily an inverse relation between virus dose and inhibitory titre over the lower range of virus concentration. With a low virus dose, e.g. two agglutinating units, partial inhibition may be observed to a relatively high titre and a sharp endpoint is difficult to obtain. A similar phenomenon has been observed by Doctor C. M. Chu (personal communication 1950) with "normal" inhibitor in mouse sera against certain influenza virus strains.

Titration of blood-group activity. These titrations were carried out with standard human anti-A sera according to the usual agglutination-inhibition technique.[30]

Analytical methods. Nitrogen was determined by the micro-Kjeldahl method using the apparatus of Markham; P by the method of Briggs[4] reducing sugar by the method of Somogyi,[36] N-acetyl-hexosamine and hexosamine by a modification of the methods described by Morgan and Elson[29] and Elson and Morgan,[11] respectively, and methyl pentose by the method of Dische and Shettles[10] using a "Uvispek" spectrophotometer.

Paper chromatography for amino acids was carried out according to the methods described by Consden, Gordon, and Martin[9] and for sugars and hexosamines as described by Partridge.[32] The aniline hydrogen phthalate reagent[33] was used for identifying sugars. All chromatograms were run on Whatman No. 4 paper, the solvents being s-collidine, butanol-acetic acid, and phenol-ammonia.

Stroma. Stroma were prepared from washed human group A red cells by precipitation in the cold at pH 5.6 as described by Jorpes.[20] Packed stroma, prepared in batches of approximately 200 ml., were stored at –10° C. until 1-2 liters had been accumulated. They were then thawed, pooled, and treated as described in the following sections.

EXPERIMENTAL

Preparation A 1. Stromata (1 liter) was added to 2 liters of pentane with vigorous stirring. The emulsion was shaken for several hours daily for four weeks and then passed through a Sharples Supercentrifuge; the supernatant fluid, which contained neither virus inhibitor nor blood-group substance, was discarded. The deposited stroma gel was collected and resuspended in 2 liters of pentane, shaken for a further ten days, and again passed through the Supercentrifuge. The stroma gel, which still contained a considerable amount of pentane, was suspended in 2.5 liters of 50% alcohol. After shaking for several hours, a finely divided stable colloidal dispersion was formed, which, after standing for 14 days, was passed four times through the Sharples centrifuge. The clear, orange-colored supernatant fluid (extract A 1-1) was concentrated to small volume *in vacuo* and stored at –10° C. The stroma deposit, resuspended in 2 liters of 50% alcohol, was again extracted for seven days and then passed through the centrifuge as before (extract A 1-2). The concentrated supernatant fluids were freeze-dried, extracted with absolute alcohol in a Soxhlet apparatus, and washed with ethanol, alcohol-ether, and acetone: yield, 33.3 gm. from extract A 1-1 and 4.2 gm. from extract A 1-2. The stroma residue was again extracted with 10% alcohol for several days, but no further active material was recoverable.

Preparation A 2. Pooled stromata (2 liters) was shaken for three weeks with 9 liters of pentane and the gel-like mass extracted successively with 15 liters (extract A 2-1) and 12 liters (extract A 2-2) of 50% alcohol over a period of several weeks. The supernatant fluids were concentrated *in vacuo* to approximately 300 ml. and centrifuged on the "Servall" angle centrifuge at 8,000 r.p.m. to remove any finely divided insoluble particles. Active virus-inhibitory material was precipitated from the slightly opalescent supernatant fluids as described below.

ETHANOL FRACTIONATION OF THE EXTRACTS

Preparation A 1. The first freeze-dried extract (A 1-1) was taken up in 400 ml. of water, stirred for one hour, and centrifuged. The active material was precipitated from the supernatant fluid by the addition of 900 ml. of ethanol in the presence of potassium acetate. After dialysis and freeze-drying 3.3 gm. of brown powder were obtained. A second fraction, collected by increasing the ethanol concentration to 80%, consisted of dark

brown tarry inactive material. The second extract (A 1-2) was fractionated with ethanol at the same level, yielding 0.7 gm. of dark brown powder.

Preparation A 2. Ethanol (1 liter) was added with stirring in the presence of potassium acetate to 700 ml. of the pooled concentrated extract A 2-1. The heavy flocculent precipitate was collected on the centrifuge, washed twice with 90% ethanol, and dried *in vacuo*—weight 43 gm. Extract A 2-2 was similarly treated with 1.8 volumes of ethanol and yielded 3.2 gm. of dark brown material. All the antihemagglutinin activity was recovered in these precipitates.

TABLE 1. ANALYSIS AND VIRUS INHIBITORY ACTIVITY OF FOUR STROMA FRACTIONS PRECIPITATED WITH ETHANOL

	Analysis			*Inhibitory activity*	
Material	*N%*	*P%*	*Hexosamine %**	*H.LEE†*	*H.MEL‡*
Fraction A1-1	9.1	1.5	10.0	1200	1280
Fraction A1-2	10.1	1.5	8.8	2000	2560
Fraction A2-1	9.6	1.0	10.0	1000	1280
Fraction A2-2	10.5	0.7	10.6	1200	1800

* Calculated as glucosamine-HCl.

† Titre of 1% solution against 10 units of indicator LEE virus.

‡ Titre of 1% solution against 10 units of indicator MEL virus.

Analytical figures for the four alcohol-precipitated fractions are given in Table 1, together with inhibitory activities against two indicator virus strains. It will be seen that the nitrogen content of the four preparations is reasonably uniform, varying from 9.1 in A 1-1 to 10.5 in A 2-2. All contain both hexosamine and phosphorus in contrast to the findings of deBurgh *et al.*[5] for ether-extracted receptor-substance. In our hands, however, preparations made by the ether-extraction process at a comparable stage of purity contained 3.4% N, 5.6% hexosamine, and 1.3% P.[27] Active fractions isolated by differential centrifugation contained 8-13% N and 1-1.5% P.[19] All four preparations inhibit hemagglutination by indicator LEE and MEL viruses to equivalent titres, the second of each pair of fractions tending to be more active than the first against both virus strains. As has been observed with most partially purified erythrocyte extracts, indicator MEL virus is inhibited to higher titre than indicator LEE, although in this case the difference is relatively small.

As the various fractions were similar in composition and activity, the three main ones (A 1-1, A 1-2, and A 2-1) were mixed, resuspended in 750 ml. of 50% ethanol, shaken at intervals for ten days, centrifuged, and the insoluble residue again extracted with 50% ethanol three times. The alcoholic extracts were pooled, centrifuged at 8,000 r.p.m. for 30 minutes, and the clear, straw-colored supernatant fluids concentrated *in vacuo* and dried from the frozen state. The material (6.8 gm.) was completely dissolved in 70 ml. of distilled water, and two volumes of ethanol were added cautiously at 0° C., a trial fractionation on a small aliquot having shown that the active component precipitated uniformly between 1.25 and 1.75 volumes of ethanol. The flocculent precipitate was collected on the centrifuge, redissolved in water, dialyzed against several changes of distilled water for 48 hours at O° C., and finally freeze-dried. The yield was 2.8 gm. of very pale yellow material (AP-1).

At this stage the titre of a 1% solution of the partially purified mucoprotein was 4,800 against H.LEE and 3,500 against H.MEL. Considerable loss of activity had, therefore, occurred during the later stages of the purification process, since the weight of active material had been reduced from 41 gm. to 2.8 gm. with only an approximately two-fold increase in activity. Further, antihemagglutinin titre against H.MEL was now slightly lower than against H.LEE. Purification was continued, however, since the rigorous chemical treatment was designed to obtain a homogeneous preparation rather than one of exceptional inhibitory activity.

Treatment with acid alcohol-ether. A portion (2 gm.) of fraction AP-1 was dissolved in 40 ml. of distilled water and added drop by drop to 200 ml. of 60-40 alcohol-ether containing 0.5% hydrochloric acid and maintained at –40° C.[28] Stirring was continued for four hours, after which time the insoluble material was removed by centrifugation at 0° C. and redissolved in 30 ml. of cold distilled water. The extraction process was then twice repeated. Finally, the extracted material was dialyzed against several changes of distilled water at 4° C., spun in the angle centrifuge at 8,000 r.p.m. for 30 minutes, and freeze-dried. The final product (AP-2) weighed 1.6 gm. The virus-inhibitory activity was not reduced by this treatment.

Treatment with the Sevag technique. A portion (0.5 gm.) of fraction AP-2 was exhaustively treated by a modified Sevag deproteinization process. The material was dissolved in 50 ml. of 0.2 M acetate buffer at pH 5.7 and stirred vigorously with 20 ml. of chloroform-butanol for one hour. The white colloidal deposit of denatured protein was removed by centrifugation, and the process continued until no further protein came out

of solution. Approximately 15 treatments were required. The supernatant fluid was then dialyzed free from buffer at 4° C. and freeze-dried, yielding 120 mg. of pure white material which dissolved rapidly to give a colorless, faintly opalescent solution. The denatured protein was shaken for several days in a large volume of buffer, centrifuged, and the supernatant fluid dialyzed and freeze-dried; 102 mg. of whitish powder was so obtained. A second 0.5 gm. portion of AP-2 was treated in the same way with the exception that the pH of the acetate buffer was 6.7 instead of 5.7.

TABLE 2. ANALYSIS OF ERYTHROCYTE MUCOPROTEINS AFTER TREATMENT WITH THE MILES-PIRIE AND SEVAG TECHNIQUES

Fraction	*N%*	*P%*	*S%**	*Reducing sugar†*	*Hexosamine‡*
Pool—AP-2	10.0	0.46	2.6	14.7	9.5
Sevag treatment	5.3	–	–	13.7	6.3
Sevag treatment residue	7.7	–	–	11.0	7.3

* Determined by Drs. Weiler and Strauss, Oxford.
† Hydrolysed with 0.5 N HCl for 24 hours; calculated as glucose.
‡ Hydrolysed as above; calculated as glucosamine-HCl.
– Not determined.

CHEMICAL PROPERTIES OF THE ISOLATED MUCOPROTEINS

All the fractions obtained in these experiments were readily and completely soluble in water. Before Sevag treatment, fraction AP-2 became faintly opalescent on the addition of an equal volume of 10% trichloracetic or sulphosalicylic acids. There was no precipitation on boiling or on adding picric or phosphotungstic acids, but slight precipitation occurred with 5% tannic acid and there was heavy precipitation with basic lead acetate. The biuret and ninhydrin tests were positive. After Sevag treatment the opalescence on adding trichloracetic and sulphosalicylic acids was very faint, but the other properties remained unaltered.

The analytical figures for the products obtained after extraction with acid alcohol-ether (AP-2), and after treatment by the Sevag technique, are given in Table 2.

Comparison with the analytical figures obtained for the earlier fractions (Table 1) shows that, as would be expected, the alcohol-ether treatment significantly reduced the P content without altering the other figures. The hexosamine: N ratio remained approximately 1 : 1. After the Sevag treat-

ment at pH 5.7, however, the N content was reduced by almost 50% but the hexosamine : N ratio was only slightly increased at 1 : 1.2.

As described in a later section, an active (cathode) fraction was separated from the Sevag-treated material by electrophoresis; the analytical figures for this fraction were N, 6.3%, reducing sugar, 15.3%, and hexosamine, 7.4%. The hexosamine : N ratio of 1 : 1.18 was, therefore, not significantly different from that of the Sevag-treated material before being submitted to electrophoresis.

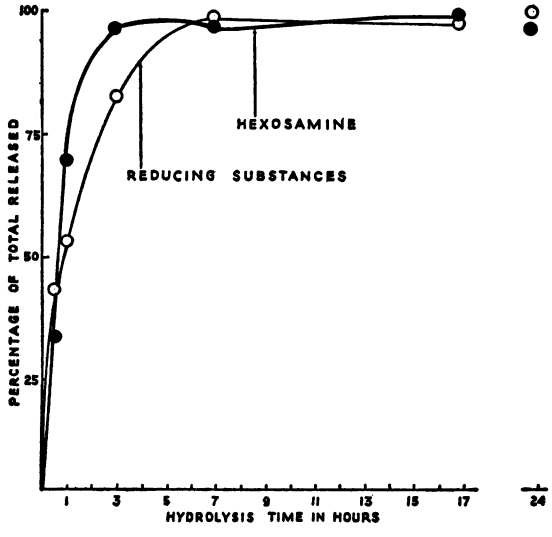

FIG. 1. Release of reducing substances and hexosamine from erythrocyte fraction AP-2 heated at 100° C. with 0.5N HCl.

A small amount of firmly bound lipid remained even after repeated acid alcohol-ether extraction. This could be demonstrated by hydrolyzing the extract with 0.5N hydrochloric acid or N acetic acid for eight hours and then extracting the hydrolysate several times with three volumes of ether and pentane. After evaporation, a small amount of fatty residue, equivalent to 4% of the original material, was recovered from the organic solvents. The nature of this lipid has not been investigated further.

Hydrolysis curve. The liberation of reducing substances and hexosamine during hydrolysis of fraction AP-2 was followed by sealing aliquots of a 1% solution in small glass ampoules with 0.5N hydrochloric acid, and immersing in a boiling water bath. Ampoules were removed at intervals and the contents carefully neutralized with sodium hydroxide. Typical results are illustrated in Figure 1. It will be seen that both reducing substances and hexosamine are released very rapidly under these conditions; the maximum value for reducing sugar is reached at 24 hours and for hexosamine at 17 hours. A considerable amount of charring takes place during hydrolysis with hydrochloric acid, and also, though to a lesser extent, with sulphuric or acetic acids.

Chromatographic examination of hydrolysate. The hydrolysis products were examined for reducing sugars, hexosamines, and amino acids by means of paper partition chromatography. In preliminary experiments considerable difficulty was encountered with severe "trailing," particularly when the papers were sprayed with the aniline hydrogen phthalate reagent for sugars. This difficulty was partly, though not entirely, overcome by the following technique: 10-20 mg. of fraction AP-2 were hydrolyzed overnight in 5N or 0.5N hydrochloric acid, and the hydrolysate then extracted twice with three volumes of ether and once with three volumes of pentane. "Trailing" was less marked when the more concentrated acid was used for hydrolysis. After centrifugation, the water layer was evaporated to dryness in a vacuum desiccator over sulphuric acid and caustic soda. The residue was taken up in one ml. of distilled water, approximately three mg. of Norite charcoal added, and the mixture brought to boiling point for one minute. After filtration the crystal-clear filtrate was again taken to dryness *in vacuo*. The residue, taken up in a small volume of water, was then applied to the paper strips and runs carried out in s-collidine, butanol-acetic acid, and phenol-ammonia with a minimum of "trailing." In one experiment the ether-extracted hydrolysate was passed through columns of the ion-exchange resins "Deacidite" and "Amberlite 1RC-50H" instead of being heated in the presence of charcoal, but the chromatographic results were inferior to those obtained after charcoal treatment.

In the final runs, two aliquots of hydrolysate were run on each paper together with "known" mixtures of galactose, glucose, glucosamine-hydrochloride, and chondrosamine-hydrochloride. The papers were then cut in two, one half being sprayed with the aniline hydrogen phthalate reagent, and the other half with the hexosamine reagents. Two pink spots with R_F values equal to those of the "known" glucosamine and chondrosamine hydrochlorides were obtained in collidine, and two similar but poorly separated spots in butanol-ammonia; a single spot only was obtained in phenol. The addition of glucosamine and galactosamine to the hydrolysate increased the intensity of the hexosamine spots, but no additional spots appeared.

In all solvents definite spots were found with R_F values corresponding to galactose and fucose, respectively. A further faint spot with the same R_F as glucose was also observed, but as the color was distinctly different, the identity of this spot is still doubtful.

Chromatograms were also run for amino-acids after hydrolysis in 6N hydrochloric acid. Ten spots were obtained on spraying with the ninhydrin reagent, corresponding to lysine, arginine, glycine, alanine, proline, valine,

leucine, and phenylalanine. Chondrosamine and glucosamine were also identifiable on these chromatograms.

Solubility in liquid phenol and diethylene glycol. The mucoprotein (fraction A 2-1) was completely soluble in 95% liquid phenol. A 1:1 mixture of liquid phenol and ethanol was cautiously added to the solution, but only approximately 10% of the material was precipitated when the ethanol concentration reached 50%. The activity of this precipitated fraction was slightly less than that of the original mucoprotein. Fractions precipitated above 50% ethanol concentration were largely insoluble in water. The exceptionally high solubility of the mucoprotein in liquid phenol is paralleled by the behavior of the sheep salivary mucoprotein.[26]

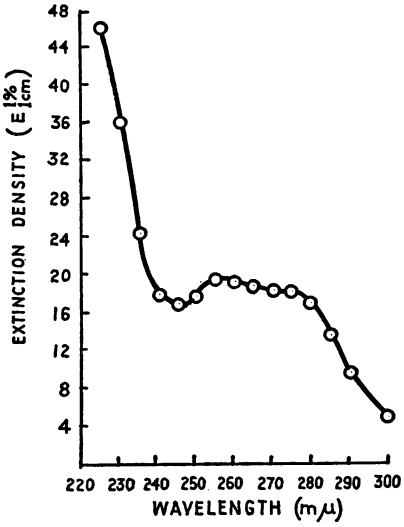

FIG. 2. Ultraviolet absorption spectrum of fraction AP-2 (0.04% aqueous solution).

The erythrocyte mucoprotein was also completely soluble in diethylene glycol, a solvent used extensively for the purification of bacterial mucoproteins. After high-speed centrifugation and passage through a Berkefeld candle, the clear solution was fractionated with ethanol at various levels between 30% and 80% final concentration. A small amount of material only was precipitated up to the 40% level, and fractions precipitated above this level were completely inactive. From these experiments it was clear that neither phenol nor diethylene glycol could be used in further purification of receptor-substance mucoproteins.

PHYSICAL PROPERTIES OF THE MUCOPROTEIN

Ultra-violet absorption spectrum. The ultra-violet absorption of fraction AP-2 was examined in the "Uvispek" spectrophotometer, using a one cm. cell and an 0.04% solution in distilled water. The curve (Fig. 2) shows a broad, ill-defined peak extending over the range 245-280 mμ. It is probable that the absorption at 245-260 mμ represents nucleoprotein; absorption from 270-280 mμ is probably due to aromatic amino acids.

*Sedimentation.** A 1% solution of extract AP-2 before and after Sevag treatment was examined in the ultracentrifuge at pH 8 in phosphate buffer (I = 0.1 + 0.15M NaCl) and at pH 4 in acetate buffer. The material sedimented very slowly at both pH 4 and 8, showing a fairly well-defined single symmetrical peak with little evidence of polydispersion. The peak appeared rather more sharply defined after Sevag treatment (Fig. 3).

*Electrophoresis.** One per cent solutions of fraction AP-2 before and after Sevag deproteinization were examined at pH 4 and 8 in the Tiselius apparatus. Before deproteinization, three broad, ill-defined components were

FIG. 3. Sedimentation diagram of 1% solutions of fraction AP-2; (a) before Sevag treatment; (b) after Sevag treatment. Phosphate buffer pH 8, I = 0.1 + 0.15M NaCl. Exposures taken 40 min. after reaching full speed, 60,000 r.p.m. (240,000 G).

FIG. 4. Electrophoresis diagram of 1% solution of fraction AP-2 after Sevag treatment. Phosphate buffer pH 8, I = 0.2. Exposure taken after separation for 300 mAH.

observed. Two well-defined and widely separated peaks were observed after Sevag treatment (Fig. 4). It was possible to separate these peaks in the electrophoresis apparatus, and, as shown in the following section, activity was associated with the larger slower-moving component only.

BIOLOGICAL PROPERTIES OF THE EXTRACTED MUCOPROTEINS

Virus inhibition spectrum. The "inhibition spectrum" of various fractions against a range of indicator and active influenza virus strains is given in Table 3.

Fraction AP-2 (pooled fractions soluble in 50% ethanol, after extraction with acidified alcohol-ether), showed the highest virus-inhibitory activity of all fractions obtained in the present experiments. The titre of 10,000 against indicator LEE was comparable to that of purified serum mucoid fractions,[22] but considerably lower than the activity of purified mucoproteins isolated from sheep salivary glands.[26] The titre of fraction AP-2 against H.LEE actually increased after treatment with alcohol-ether, a phenomenon not yet

* These procedures were carried out through the kindness of Dr. R. A. Kekwick.

explained, but observed also with serum mucoid[22] and erythrocyte mucoproteins extracted in ether. A significant increase in inhibitory activity was observed against all indicator virus strains as compared to active (unheated) virus: in the case of LEE and indicator LEE an approximately 20-fold increase occurred with each fraction shown in the table. Another point to be noted is that, with these preparations, the titre against H.MEL was lower than that against H.LEE.

In contrast to the result expected from previous experience with receptor analogues, very extensive loss of inhibitory activity against all strains occurred after Sevag treatment at either pH 5.7 or 6.7: the titre against H.LEE, for example, was reduced to approximately 20% of the original

TABLE 3. ANTIHEMAGGLUTININ ACTIVITY OF 1% SOLUTIONS OF ERYTHROCYTE MUCOPROTEINS BEFORE AND AFTER SEVAG TREATMENT

Fraction	*LEE*	*H.LEE*	*MEL*	*H.MEL*	*PR8*	*H.PR8*
Pool—AP-2	500	10,000	500	4,000	800	3,200
Pool—Sevag treated pH 5.7	10	1,750	10	100	10	10
Pool—Sevag residue pH 5.7	500	9,000	100	4,000	700	2,800
Pool—Sevag treated pH 6.7	100	2,800	10	600	80	300
Pool—Sevag residue pH 6.7	180	9,600	10	2,800	750	2,400

figure, and the activity against other strains was decreased to an even greater extent. Thus, after treatment at pH 5.7, none of the active strains were demonstrably inhibited, nor was H.PR8. As found with other erythrocyte extracts, activity against active MEL and H.PR8 is particularly sensitive to destruction during chemical treatment, while that against H.LEE is the most stable. At pH 6.7 some activity remained against active LEE and PR8, and against H.PR8, but it is clear that even the relatively milder conditions have brought about severe loss of inhibitory activity. It should be noted, however, that the soluble mucoprotein extracted from the precipitated protein gel resulting from the Sevag treatment showed activity of similar order to that of the original material (before Sevag treatment) against strains LEE, H.LEE, H.MEL, PR8, and H.PR8, but again the titre against MEL was significantly reduced.

Effect of electrophoresis. The virus-inhibitory activity of the two electrophoretically separated components from the Sevag fraction treated at pH 5.7 is given in Table 4.

The results obtained for the electrophoretically separated components are of considerable interest. The small anode component (Fig. 4) was com-

pletely inactive toward all strains, but the larger, slower-moving cathode peak was associated with the slightly higher activity against H.LEE than was the starting material. It is clear, however, that complete concentration of activity against this strain has not been achieved, since the cathode component represents only 5% of the total material (100 mg.) subjected to electrophoresis.

Inactivation by influenza virus and RDE. Sufficient data may be given here to show that the inhibitory action of the purified erythrocyte extract is progressively lost on incubation with living influenza virus or the "receptor-destroying" enzyme of *V. cholerae*[37] as has previously been described for purified ovarian cyst mucoid[6] and purified sheep salivary mucoid.[39] More

TABLE 4. VIRUS INHIBITORY ACTIVITY OF ELECTROPHORETICALLY SEPARATED ERYTHROCYTE FRACTIONS

Fraction	*LEE*	*H.LEE*	*MEL*	*H.MEL*
Sevag treatment pH 5.7	<10	1,750	<10	100
Cathode component	<10	2,500	<10	<10
Anode component	<10	<10	<10	<10

detailed studies now in progress on the kinetics of inactivation with a series of virus strains will be described in a later paper.

Aliquots of a solution of purified erythrocyte extract (AP-2) were incubated with 100 agglutinating units of fresh LEE or MEL virus diluted in acetate buffer pH 6.4 containing 0.1% $CaCl_2$. Control tubes with heat-inactivated virus and without virus were included. After two hours' incubation at 37° C. the tubes were removed from the bath and brought to 100° C. for one minute to destroy the active virus. It was found that active LEE virus reduced antihemagglutinin activity against H.LEE to 30% and against H.MEL to less than 1% of the control values. Active MEL virus reduced the titres against the two strains by 50% and 70% respectively.

Aliquots of purified erythrocyte extract were also incubated with a purified eluate of *V. cholerae* receptor-destroying enzyme (RDE) diluted in calcium acetate buffer pH 6.4. After incubation at 37° C. for two hours with an equal volume of a 0.005% solution of RDE, a solution of erythrocyte extract showing originally a titre of 6,000 against H.LEE and 4,000 against H.MEL had become completely inactive (less than 1:10) against both strains.

Inactivation by periodic acid. Like the red cell receptor[18] and the soluble Francis inhibitor in normal serum and purified serum mucoid[22] the inhibi-

tory activity of the purified erythrocyte extract is sensitive to the periodate ion. Exposure of a 1% solution of fraction AP-1 in acetate buffer at pH 5.0 to 0.005M periodic acid for one hour at room temperature reduced the titre (H.LEE) from 9,600 to 10.

Action of trypsin. A 0.5% solution of purified fraction AP-2 was incubated for two hours with an equal volume of 2% "Difco" trypsin in borate buffer pH 8.1, controls containing heat-inactivated enzyme being included. No significant reduction in titre occurred against LEE, H.LEE, MEL, or H.MEL.

Blood-group activity. The anti-A and anti-H activity of the extracts was estimated at various stages during the fractionation process. No anti-A activity was detected in the crude extracts, and only minimal (less than 1 : 10) inhibition of anti-H sera. The purified fraction AP-1 was kindly examined in detail by Miss Joan Thompson, who reported no inhibition (less than 1 : 2) of anti-B, anti-M, or anti-D sera, and inhibition titres of 64 and 512 against anti-H and anti-A sera respectively.

DISCUSSION

The receptors of influenza virus exist on the erythrocyte surface presumably as part of a lipomucoid complex. The first stage of the chemical isolation of receptor-substance in methods so far described involves preliminary treatment with a concentrated lipid solvent which releases part of the complex in an extractable form; this is achieved in the process of deBurgh *et al.*[5] by heating (or freezing and thawing) stroma with ether, and with dried red cells by prolonged extraction with 95% alcohol.[16, 27] It is probable that in both these processes only a small fraction of the total receptor-substance is released from the stroma.[19] Further purification in all processes depends essentially on removal of firmly bound lipid from the extracted lipomucoid and fractionation of the protein components.

The chief advantages of the pentane-dilute alcohol process described in this paper are, first, the virtual absence of blood-group activity which is constantly associated with erythrocyte extracts prepared by the ether extraction of stroma or alcohol extraction of freeze-dried red cells[19, 27]; and, secondly, the isolation of a product which is completely and rapidly soluble in water and which retains constant activity even after storing in the dry state for up to two years. In our hands, erythrocyte extracts prepared by other methods frequently become partially insoluble after storage *in vacuo* and may also become largely insoluble after storing in solution at –10° C. for several days. This applies particularly to preparations made by the

ether-extraction process. The final yield of active substances with the pentane method is considerably greater than with the alcohol extraction of freeze-dried cells or with the ether extraction of stroma. It is clear, however, that none of the chemical methods so far described for preparing receptor-substance even approaches the ideal.

The presence of two hexosamines in the erythrocyte mucoprotein seems established on chromatographic evidence. Two spots with R_F values equal to those of glucosamine and galactosamine were invariably found, and no further spots appeared when the two known hexosamines were added to the hydrolyzate. However, definitely to establish the identity of the two hexosamines it would be necessary to isolate crystalline derivatives from large amounts of extract—a procedure which has not yet been possible. In this connection it may be noted that the single hexosamine present in sheep salivary mucoid was found to have the same R_F value as galactosamine in various solvents, but the melting point of the dinitrophenyl derivative was distinctly lower than that of the authentic galactosamine compound.[26] The presence of galactose seems reasonably certain; this sugar has been found in all erythrocyte extracts so far examined. It is probable that galactose occurs in small amounts only, since the spots obtained were weak, usually fainter than those of fucose. The presence of fucose, which is found in small amounts in all red cell extracts so far examined, was confirmed both by chromatography and the spectrophotographic method. It is of interest that the electrophoretically homogeneous mucoprotein of human urine,[40] which also actively inhibits influenza virus hemagglutination, contains glucosamine, galactosamine, galactose, mannose, and fucose.[14a]

The question of homogeneity of this, and in fact in all preparations of large molecules, is a difficult one; electrophoresis and ultracentrifugation provide some evidence of the range of variation in mobility and molecular size, but one is always faced with the possibility of dealing with mixtures of molecules of different biological specificity which show similar physical behavior in an electrical or sedimentation field. The present preparation shows a single peak even after prolonged sedimentation in the ultracentrifuge, little evidence of polydispersion, and extremely slow sedimentation, thus indicating the presence of small molecules of the same order of size: the molecular weight is probably less than 87,000 since the erythrocyte mucoprotein moves more slowly than the homogeneous mucoprotein isolated from sheep salivary glands.[26] The electrophoretic pattern before Sevag treatment is a complicated one with at least three differnt components; after Sevag treatment two sharp, well-separated peaks are obtained, inhibitory activity (against H.LEE) being associated with the considerably

larger slow-moving peak only. In this instance, therefore, an electrophoretically and ultracentrifugally homogeneous mucoprotein has been isolated from erythrocytes with moderate antihemagglutinin activity against one virus strain.

The extensive and unexpected loss of activity against all virus strains after mild Sevag treatment is difficult to interpret. In previous studies on the serum mucoid "Francis inhibitor" it had been found that antihemagglutinin activity against H.LEE was lost only if Sevag treatment was carried past a certain stage, but that activity against certain influenza A strains ("non-specific inhibitor") was destroyed very rapidly. These findings, together with other differences, were considered by McCrea[22] to establish the non-identity of the Francis and non-specific inhibitors, the former being associated with serum mucoid and the latter with the more labile serum proteins. This problem has also been discussed by other workers (e.g. Smith, Wilson, and Westwood[35]) who consider that it is more probable that the two inhibitors exist as different components of one complex. It is possible that Sevag treatment in the present instance has inactivated one part of a large molecule (that part inhibiting active and indicator A strains) while leaving the more stable component inhibiting H.LEE relatively intact. The experimental evidence obtained in the present investigation is against this interpretation, however, since quantitative recovery against the other strains (with the exception of MEL) was obtained from the deposited protein gel. Thus, it would appear that a *separation* of a nonprecipitated H.LEE inhibitory component has been obtained, and not simply inactivation of one component or group of components in a complex molecule. It will be noticed that the active material washed out from the protein gel resulting from Sevag treatment is similar in composition to the fraction remaining in solution (Table 2), the hexosamine : N ratio being practically unchanged. This suggests that one is dealing with a mixture of chemically very closely related mucoproteins, the chief distinction being that the inhibitor of H.LEE is less readily precipitated by chloroform.

The virus "inhibitor spectrum" of the pooled material, before Sevag treatment, is H.LEE > H.MEL > H.PR8 > PR8 > LEE=MEL. In the majority of erythrocyte extracts prepared by other methods, inhibitory titre against H.MEL is approximately twice as high as against H.LEE, the reversal in the present instance probably being a reflection of the rigorous chemical treatment. In the "crude" extract from pentane-treated stroma the titre against these two indicator strains is scarcely significantly different. It is perhaps impossible to compare the activities of erythrocyte extracts prepared by different methods, since the relative degree of purity is difficult

to assess; however, a 1% solution of the most active mucoprotein fraction isolated by the ether-extraction process inhibits H.LEE to a titre of 16,000 and H.MEL to 56,000; the corresponding figures for the most active alcohol-extract from dried red cells are 8,000 and 16,000 respectively.[25] The pentane preparation, therefore, shows intermediate activity against H.LEE but is less active than those isolated by other methods against active and indicator A strains.

As would be expected, the isolated receptor-substance is inactivated after contact with living virus or with the bacterial receptor-enzymes. Inactivation by virus is slower than has been found with other erythrocyte preparations, a finding which will be discussed in detail in a later publication dealing with the interaction of virus and isolated red-cell receptor substances. The resistance to inactivation by trypsin should be mentioned here, however, since Hirst[18] stated that trypsin destroyed receptors on the erythrocyte, although in preliminary work on receptor-destroying enzymes we had not found this to be so.[7, 27] The experimental details in Hirst's paper are not entirely clear, however, no indication of enzyme concentration or time of incubation being given. In cases where trypsin enzymically inactivates receptor analogues such as sheep salivary mucoid, it does so at extremely high dilution.[26] None of the erythrocyte extracts we have prepared are significantly affected even on incubation with 1% trypsin for several hours. Howe[19] found that the antihemagglutinin activity of a "lipid-rich" stroma fraction resisted proteases, but a "lipid-poor" fraction was susceptible to chymotrypsin.

Finally, the relatively slight blood-group activity of this preparation is noteworthy, since all other erythrocyte extracts prepared in our laboratory show high activity toward the specific group from which the cells are derived, and also with regard to the heterogenetic "H" blood-group substance.[31] The specific blood-group activity of the purified pentane-treated extract is at most only 2%, and the "H" substance activity is considerably less than 1%, of the titres shown by erythrocyte preparations obtained by ether or alcohol extraction without preliminary pentane treatment (W. T. J. Morgan and W. M. Watkins, in preparation). From the data at present available it is difficult to decide whether preliminary pentane treatment degrades and inactivates blood-group substance, or whether this solvent, which appears to facilitate the release of virus receptor-substance, actually "fixes" blood-group substance to the stroma in a form which is not extractable in 50% alcohol. A similar finding with freeze-dried red-cells treated with pentane will be described in the next paper in this series. The choice of solvent used in the preliminary extraction obviously is of great importance

in attempting to obtain receptor-substance free from blood-group substance, since in no case has it proved possible chemically to separate the two substances when both are present in the one solution.

SUMMARY

A method is described for the isolation of influenza virus "receptor-substance" from human erythrocytes. Red cell stroma were shaken with pentane to form a colloidal gel from which the active substance was extracted in 50% ethanol.

A stable virus-inhibitory mucoprotein has been obtained which was completely soluble in water, 95% phenol, and diethylene glycol. The analytical figures were: N 10.0%, P 0.5%, S 2.6%, reducing sugar 14.7%, and hexosamine 9.5%. Chromatography revealed the presence of two hexosamines (probably glucosamine and galactosamine), galactose, fucose, and amino acids. Approximately 4% of ether-soluble lipid was released on hydrolysis with acid. The ultraviolet absorption spectrum suggested the presence of nucleic acid.

One very slow-moving component with only slight evidence of polydispersion was found in the ultracentrifuge. At least three components appeared on electrophoresis. After deproteinization by the Sevag technique, two sharply defined peaks were found on electrophoresis, the larger and slower-moving one alone being associated with virus inhibitory activity against indicator LEE virus.

A 1% solution of the extract inhibited hemagglutination by heated LEE virus to a titre of 10,000. The inhibition "spectrum" was H.LEE > H.MEL > H.PR8 > PR8 > LEE=MEL. After Sevag treatment approximately 80% of the activity against H.LEE and practically all activity against active and indicator A strains was removed from solution. Inhibitory activity against all strains, with the exception of MEL, could, however, be quantitatively recovered from the deposited protein-mucoprotein gel.

Capacity to inhibit virus hemagglutination was lost when the mucoprotein was incubated with active virus, or with the receptor enzyme of *V. cholerae*. Incubation with 1% trypsin did not significantly reduce the antihemagglutinin activity.

The blood-group activity of the mucoprotein was at most only a few per cent of that found in extracts prepared by other methods.

The relation of this mucoprotein to those prepared from erythrocytes by alternative methods, and to soluble "receptor analogues," is briefly discussed.

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