PREPARATION FROM HUMAN RED CELLS OF A SUBSTANCE INHIBITING VIRUS HEMAGGLUTINATION*

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Experiments on the inhibiting effect of human and sheep red blood cell extracts on the hemagglutinating action of mumps and influenza viruses have been described in a previous report (1). Results were presented which suggested that the inhibiting agent found in these red cell extracts was a derivative of, or identical with, the material which in the intact cell constitutes the receptor site for the virus in the hemagglutination reaction. These results consisted partly in a demonstration that, in the systems studied, the species specificity of the inhibition reaction paralleled that of the hemagglutination reaction. Thus it is known that influenza (PR8) virus will agglutinate human and chicken red cells, but not sheep cells; and that mumps virus will agglutinate all three types of red cells. It was found that the human red cell extracts inhibited the agglutination of human and chicken cells by influenza virus; and that of human, chicken, and sheep red cells by mumps virus. Extracts of sheep cells, however, inhibited the agglutination of sheep cells by mumps virus, but did not inhibit agglutination of human and chicken cells by influenza virus. Further support for the view above mentioned was provided by the discovery that in a mixture of virus and inhibitor at 37°C. the latter was inactivated, just as is the receptor of intact cells under similar conditions in the elution phenomenon described by Hirst (2).

Recently three other laboratories have reported interesting data in relation to the effect of various tissue and cell extracts and other substances on virus hemagglutination and multiplication. Friedewald, Miller, and Whatley (3) described the hemagglutination-inhibiting effects of saline extracts of various human and animal tissues and red cells. Horsfall and McCarty (4) produced evidence to show that certain bacterial and plant polysaccharides seem capable of interrupting the multiplication of PVM virus in mouse lung. The intrapulmonary multiplication of virus was reflected by a rise in hemagglutination titer of triturated infected mouse lung. Administration of the polysaccharides diminished or prevented this increase in titer after infection. Green and Woolley (5) have demonstrated the inhibitory effect of various animal and

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plant polysaccharides on the hemagglutination of chicken red cells by influenza PR8 virus, and on the multiplication of the same virus in the chicken embryo.

The inhibitor previously reported from this laboratory was designated as a lipid extract of red cells. The present report deals with further experiments on methods of preparation of the soluble inhibitor, on its partial purification, and on some chemical properties of the most active preparations that we have thus far obtained.

EXPERIMENTAL

Assay.—For assay purposes one unit of inhibitor was designated as that amount of material which would completely inactivate two minimal hemagglutinating doses of influenza virus, strain PR8. The minimal hemagglutination dose was previously determined by titration as the smallest amount of virus which would agglutinate 0.1 cc. of 2.5 per cent cells in a final volume of 0.5 cc. Unless otherwise stated, human group O cells and chick embryo allantoic fluid infected with PR8 influenza virus were used in the assays. In the actual test 0.2 ml. of twofold serial dilutions of the extract to be tested was mixed with two minimal hemagglutinating doses of virus contained in 0.2 ml. of saline buffered to pH 7.4 by addition of 10 per cent of 0.15 molar phosphate buffer. The mixture was kept at 4°C. for one-half hour and then 0.1 ml. of a 2.5 per cent suspension of red cells in buffered saline was added, the mixture was shaken, and the cells were allowed to settle in the cold room. The degree of agglutination was read by the pattern of the sedimented red cells after the method of Salk (6). The titer represents the reciprocal of the highest dilution of inhibitor showing complete absence of agglutination.

Extraction of Inhibitor from Red Cells.—The first step was the conversion of cells[1] to stroma.[1] In earlier experiments the inhibitor was extracted from pools containing only group O cells. It is therefore evident that although it has been reported that blood group specific substance A exerts an inhibitory effect on hemagglutination by PR8 virus (5), the inhibitor under consideration is not the A or B antigen. This is further substantiated with respect to the A antigen by the fact that a highly purified specimen of group A substance from hog stomach[2] did not inhibit hemagglutination in a concentration of 1 mg. per cc. It is also of interest that a preparation of hog's stomach group O substance[2] did not inhibit hemagglutination at a concentration of 250 gamma per cc. Friedewald (3) has also reported group A substance to be inactive as an inhibitor of chicken cell agglutination by influenza virus. Owing to the difficulty of securing adequate amounts of blood the later experiments were done with pools of all the types. There was no significant difference in the amount or quality of extractable inhibitor obtained.

In the earlier experiments the cells were washed three times with saline. Since inhibitor material from unwashed cells was the same with respect to yield and behavior as that obtained from washed cells, and since samples of pooled plasma showed no inhibitor extractable by the method outlined below, this tedious step was omitted in later preparations.

The stroma was prepared after the method of Jorpes (7). The packed cells were lysed by the addition of ten volumes of cold (5°C.) distilled or tap water, and the pH was brought to 5.6 by the addition of 1 per cent HCl or acetate buffer pH 4.0 (700 ml. 10 N HAC, 200 ml. 7 M NaAC,

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[1] Cells and stroma were kindly supplied by the Antitoxin and Vaccine Laboratory, Massachusetts Department of Public Health, and by the Sharp and Dohme Company, Glenolden, Pennsylvania.

[2] This was kindly made available to us through the courtesy of Dr. E. A. Kabat, Columbia University, College of Physicians and Surgeons.

400 ml. distilled water). The stroma settled out in a thick curd which was separated in the Sharples centrifuge. The pink precipitate was diluted with distilled water to a volume roughly one-fifth that of the original, packed red cells, and the pH of the thick fluid was brought to 7.1–7.2 by the addition of 0.5 N NaOH with vigorous stirring.

Calvin *et al.* (8) have shown that stroma prepared as described consists of two fractions, elinin and stromatin, separable by high speed centrifugation over a pH range of 7.5 to 7.8. It was of interest to determine which of these two fractions carried the inhibitory activity.

Stroma was suspended in buffered saline pH 7.4 to give a pinkish opalescent solution. On spinning this suspension at 18,000 R.P.M. for 30 minutes, the elinin was found as a pinkish grey pellet on the bottom of the tube. The clear supernatant stromatin solution was pipetted off, the elinin was resuspended in the original volume of buffered saline, and the two solutions were assayed for activity.

The titer of the stromatin solution was less than twenty, while that of the elinin suspension was 2500. Obviously the activity is associated with the elinin fraction.

Various attempts to resolve the elinin particles into soluble components with preservation of activity were unsuccessful until finally the fact that Calvin *et al.* had reported it to be a lipoprotein suggested the use of the McFarlane (9) procedure for dissociating lipoproteins. This consisted of alternate freezing and thawing of an aqueous suspension or solution of the material in the presence of diethyl ether. The procedure when applied to stroma yielded the inhibitor in an ether-soluble form.

Whole stroma paste, pH 7.0, was distributed in 70 ml. amounts into 250 ml. bottles. One and one-half to two volumes of peroxide-free ether were added to each bottle, which was then stoppered and shaken vigorously until an emulsion had formed. The unstoppered bottles were then placed in a dry ice box until the emulsion was frozen. After thawing, the bottles were stoppered, shaken vigorously, centrifuged, and the ether was then siphoned off. Fresh ether was added, and the procedure was repeated six times. On further extraction the yield of ether-soluble active material decreased although the inhibitor content of the stroma was not entirely exhausted. Similar results were obtained in the extraction of lung tissue (Fig. 1).

The combined pale yellow ether solutions were evaporated *in vacuo* under a stream of CO_2 to a light brown mud which was kept under suction until most of the water was removed. This was then thoroughly triturated with acetone (200 ml. per liter of starting stroma suspension) and the supernatant was separated by centrifugation and decantation. The extraction was repeated two or three times until the acetone washings were colorless. The same process was repeated with 95 per cent alcohol, using approximately 75 ml. of alcohol per liter of stroma for each extraction, until the alcohol washings were colorless. It was found that not only was all the activity left in the acetone- and alcohol-insoluble fraction, but that the inhibitory titer was actually increased by the passage into the acetone and alcohol of some material which antagonized the inhibitor (Table I). This antagonistic effect is probably due to the action of some component of the acetone- and alcohol-soluble fraction on the cells, since this fraction, when suspended in saline, caused agglutination of the cells in the absence of virus.

Following the acetone extraction, the residue remained in the form of a light brown dry powder, whereas it is a gummy mass after extraction with alcohol. Therefore it was found

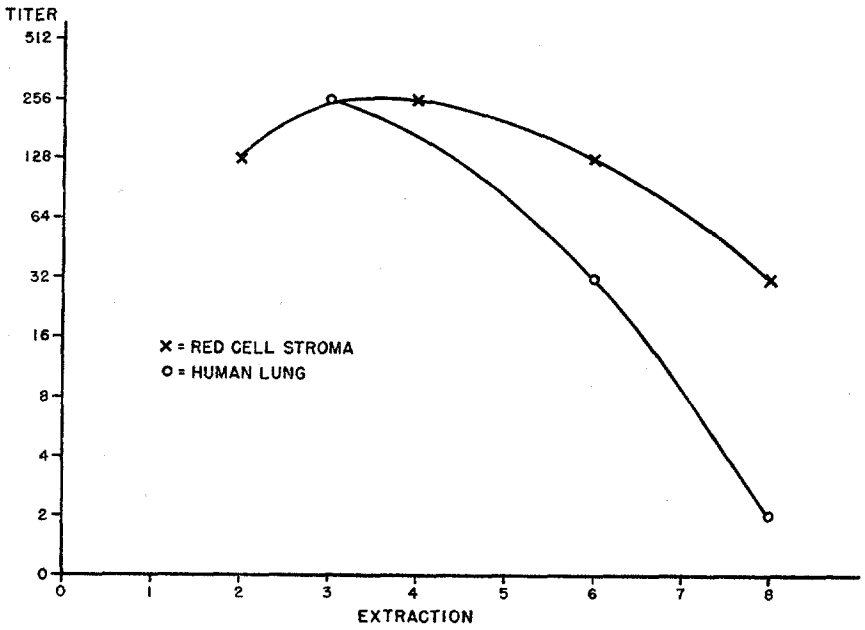

FIG. 1. Titer of successive extractions of human lung and red cells.

TABLE I

Removal of Inhibitor Antagonist by Acetone and Alcohol Extraction

	Dilution of inhibitor								
	2	4	8	16	32	64	128	256	512
A	−	+++	+++	+++	+++	+++	+++	+++	+++
B	−	−	−	−	−	−	−	−	+++
C	−	+++	+++	+++	+++	+++	+++		
D	−	−	−	−	++	+++	+++		
E	−	−	−	−	−	+	+++		

A, batch 14. Residue of 100 ml. ether extract resuspended in 6 ml. saline.

B, batch 14. Residue of 100 ml. ether extract treated with acetone and alcohol and resuspended in 6 ml. saline.

C, batch 28. Residue of 100 ml. ether extract resuspended in 3 ml. saline.

D, batch 28. Residue of 100 ml. ether extract treated with acetone and resuspended in 3 ml. saline.

E, batch 28. Residue of 100 ml. ether extract treated with acetone and alcohol and resuspended in 3 ml. saline.

convenient to treat the alcohol residue once again with acetone to obtain the active insoluble material in the powdered form. Activity at this point was about 0.1 unit per gamma, and the yield averaged 2 to 3 gm. per liter of original stroma. This powder was suspended in chloro-

form overnight, then filtered through a sintered glass filter of medium porosity, washed, and dried. A considerable amount of substance, usually about 50 per cent, dissolved in chloroform to give a dark brown solution, and approximately 15 per cent of the activity was often found in this soluble fraction. The chloroform-insoluble brown powder contained most of the inhibitor and its activity was of the order of 0.16 unit per gamma.

The active material was water-soluble and could be extracted almost completely by allowing the powder to soak in water overnight in the refrigerator, or more rapidly by heating an aqueous suspension of the powder at 60°C. for 20 to 30 minutes in a water bath. On centrifuging the water-insoluble material at 18,000 R.P.M. (25,000 *g*) in the high speed angle head centrifuge for 30 minutes, clear, light brown aqueous solutions were obtained. The activity of these solutions was of the order of 1.25 units per gamma of dry weight. The active solutions gave a positive Molisch test and contained 7 per cent N. The active material was apparently a substance of high molecular weight as it was non-dialyzable. It was sedimentable in the ultracentrifuge at 200,000 to 240,000 *g* but was obviously impure, as the solution showed several components in the analytical ultracentrifuge. About one-quarter of the material appeared to be a polydisperse fraction sedimenting very readily in the ultracentrifuge (sedimentation constants greater than 15 S). Slightly more than half of it sedimented fairly uniformly, with sedimentation constants of 10 to 12 S. The remainder was found in two minor components sedimenting more slowly. The active component was in the more rapidly sedimenting fractions, and probably in the one coming down first. This is only a tentative conclusion as a clear separation of the two most rapidly sedimenting fractions was not achieved. Further purification of the fractions was not attempted since it was found possible to prepare decidedly more active material by another method, as follows:—

Preparation of Chloroform-Soluble Material.—The freezing and thawing of the stroma-ether mixture was cumbersome, and a more convenient method of extraction was desired. This was achieved by heating a neutral stroma paste containing a few per cent of ether at 70°C. for 15 minutes. The mixture was cooled to room temperature afterwards and extracted with approximately 3 to 4 volumes of ether, letting it remain at room temperature for 24 hours with frequent shaking. Occasionally a relatively stable emulsion was formed on shaking, which broke only on addition of more ether, or after prolonged standing. The extraction was repeated three times. Further extractions resulted in decreasing amounts of activity.

After removing the ether by distillation *in vacuo* the residue was dried and treated with acetone and alcohol as previously described. At this point it was found that the activity lay in the insoluble light brown residual powder as before, but that, in contrast with the findings at the corresponding stage in the previous methods of preparation, this powder was almost completely soluble in chloroform. On treating it with this solvent, there remained a slight amount of insoluble brown gum, possessing some activity, which was removed by filtration through a sintered glass funnel. The resulting clear, amber-colored fluid contained about 90 per cent of the activity.

Addition of seven volumes of acetone to the filtrate gave a yellow, flocculent precipitate containing all of the activity. After decanting the supernatant the precipitate was dissolved in a minimal amount of chloroform and one and one-half volumes of acetone was added. A light flocculent precipitate separated out. This contained 80 per cent of the activity. A moderate amount of dark brown impurity remained in the supernatant soluble portion, which was decanted after centrifugation. The precipitate was redissolved in a minimal amount of chloroform and reprecipitated with one and one-half volumes of acetone. It dried to an amber-colored solid which slowly dissolved in water to give a ropy solution. The activity of this solution was approximately 0.3 unit per gamma. It contained lipid, as evidenced by the appearance after a short period of hydrolysis of an acid-insoluble, ether- and alkali-soluble oil. There was very little of this lipid however, and no substance possessing activity could be extracted from the untreated, aqueous solution, on shaking with ether.

The marked difference in chloroform solubility between the inhibitor solution obtained by the first and second of the methods described was of some interest, since it presented the possibility that two different substances were involved. However, it was found that the chloroform-soluble inhibitor could be converted into the chloroform-insoluble form by freezing and thawing an aqueous solution in the presence of ether and a high partial pressure of carbon dioxide. Without this last the conversion was not effected. It was also found that ether extraction of the thoroughly dried material for 5 or 6 days at room temperature was partially effective in converting the powder to the chloroform-insoluble form. It seems likely that there was a lipid complex involved, the dissociation of which was subject to a pH effect. This possibility was supported by one experiment in which it was found that conversion of the chloroform-soluble active material to the chloroform-insoluble form had occurred after evaporation of a small amount of the former to dryness by means of a stream of CO_2. The point, however, has not been thoroughly investigated.

Conversion of the Chloroform-Soluble to the Insoluble Form.—A 0.390 gm. sample of chloroform-soluble powder containing 125,000 units of activity was dissolved in 10 ml. of water and shaken with an approximately equal volume of ether. No color went into the ether solution. The mixture was frozen and thawed under the conditions noted above. On shaking after each thaw the ether layer was seen to become increasingly colored. Following the sixth thaw the ether was siphoned off and the water layer was repeatedly extracted with portions of ether until no more color went into the ether layer. At this point the water layer was faintly straw-colored. The water was removed by lyophilization, the residue was extracted with ether, chloroform, and petroleum ether, and dried, providing an almost white powder. On resuspending in 4 ml. of water, this powder went slowly into solution after swelling into jelly-like particles. A small amount of water-insoluble material was separated by centrifugation. The clear viscous supernatant had a solid content of 12.0 mg. per ml., and had an activity of 2.5 units per gamma against both mumps and PR8 viruses.

The nitrogen content of this material, determined by a micro modification of the Nessler method (10) was 2.6 per cent. It gave a positive ninhydrin test. There was no inactivation on prolonged shaking with chloroform. A quantitative determination showed no glucosamine to be present.[3] Further information on the nature of the nitrogenous component will await the preparation of more material. There was no detectable phosphorous present in a 100 gamma sample.

The material contained carbohydrate as evidenced by the presence of a positive Molisch test. The quantitative orcinol test for pentoses (11) and the Dische test (12) for uronic acids were completely negative when assayed on 100 gamma samples. Under the conditions used 10 gamma control samples of xylose and Type III pneumococcus polysaccharide showed definite color. The unhydrolyzed material gave a reducing value of 1 per cent (as glucose) by the Malmros modification of the Folin-Wu method for glucose (13). On hydrolysis

[3] This determination was kindly performed by Mr. Cava through the courtesy of Dr. Karl Meyer of Columbia University.

the reducing sugar, determined as glucose equivalents, was found to be 48 per cent. The maximum reducing value on hydrolysis with 2 N HCl was found to occur in 1 hour, after which time it slowly decreased. The inhibitor is extremely labile to acid hydrolysis, being completely inactivated by treatment with 0.1 N HCl for 5 minutes and 75 per cent inactivated by 0.001 N HCl for 10 minutes in a boiling water bath. One-thousandth normal NaOH at 100°C. for 10 minutes caused no loss of activity. A slight amount of insoluble material appeared on acid hydrolysis but the nature of this has not yet been investigated.

A 1 per cent solution showed no measurable optical activity in a 1 dm. tube.

This preparation was completely sedimentable in the analytical ultracentrifuge but showed no sharp peak, behaving as a polydisperse material with a minimum sedimentation constant of about 20. This bears out the previously described results obtained with material in which it was indicated (but not clearly demonstrated) that the active component of this impure preparation was in the most rapidly sedimentable fraction.

These purified inhibitor solutions were readily inactivated by virus. In one experiment, 12,000 units of inhibitor were inactivated by 300 hemagglutinating units of PR8 virus in 16 hours at room temperature with recovery of 30 per cent of the activity of the virus. In the absence of the virus the inhibitor proved stable in watery solution at room temperature.

DISCUSSION

From the above data it is probable that the water-soluble, chloroform-insoluble inhibitor is polysaccharide in nature. This is not surprising, especially when considered in connection with the previously mentioned findings of Horsfall, Woolley, and the still earlier reports of the inhibitory effects of polysaccharides on bacteriophage (14) and on plant viruses (15). Whether or not it is a complex protein or lipopolysaccharide it is impossible to say until further information is available on the state of purity of the active inhibitor and the chemical nature of its structural components. It is evident that the chloroform-soluble form of the inhibitor is associated with the presence of lipid material. The conditions under which the lipid can be separated from the chloroform-soluble active material indicate the possibility of a complex consisting of lipid and water-soluble, chloroform-insoluble inhibitor. There are, of course, many known types of lipoprotein and lipopolysaccharide complexes, and the nature and stability of the binding forces of these complexes show wide variation. It is possible that the active ether- and chloroform-soluble substance represents material that is less degraded than the water-soluble inhibitor, and it might, therefore, be a closer approximation to the natural state of the receptor as it exists in the red cell. On the other hand, the possibility exists that the association of lipid and inhibitor, responsible for the chloroform solubility of the latter, is merely an artifact caused by the method of preparation. Further in-

vestigations should reveal more precisely the nature and characteristics of the material under consideration.

It is of interest that our inhibitor is more active in preventing hemagglutination than any of the numerous polysaccharides from various sources and the other materials tried by Woolley. His most active preparations gave almost complete inhibition of agglutination in concentrations of approximately 100 gamma per ml. In a roughly comparable test our most active material gave complete inhibition, that is "O or trace" of agglutination by Woolley's criteria, in a concentration of 1.1 gamma per ml.

Friedewald and collaborators also described the preparation of inhibitor in high titer by a saline extraction of human red cells. In some experiments on specificity of inhibition, and elution of virus from inhibitor, they obtained data similar to our results already described (1). They also arrived at the conclusion that the active material in the extracts represented the receptor substance of the red cell. Their active extracts were prepared by lysing red cells in a Waring blendor followed by centrifugation of the mixture at 3,000 R.P.M. It would be of interest to know whether these extracts represent a soluble inhibitor or a suspension of finely divided stroma (elinin). In our experience elinin prepared by hemolysis of red cells remained suspended, with retention of inhibitor activity, after centrifugation at 3,000 R.P.M. at pH 7.0; but it was sedimented at 18,000 R.P.M. for 20 minutes to the extent of 90 per cent as estimated by the disappearance of inhibitor activity in the supernatant.

Friedewald and collaborators describe the preparation of active extracts from various animal and human tissues, and raise the question as to the identity of tissue and red cell inhibitor. In this connection it is of interest that although we have been able to obtain from human lung an ether-soluble inhibitor, we have not been able to find similar material in human liver, kidney, or serum. This and various other biological properties of the inhibitor substances are under investigation.

SUMMARY

Methods have been described for the extraction and purification of an agent inhibiting the hemagglutination of red cells by influenza (PR8) and mumps viruses. Human red cells have served as the chief source of the inhibitor but the latter has also been found in human lung.

The active extracts have been purified to the extent that 0.1 gamma of material suffices to inhibit one hemagglutinating dose of virus. Incomplete chemical characterization of the most highly purified fractions available indicates the presence of 2.6 per cent nitrogen, at least 50 per cent of polysaccharide, and no phosphorus. In the ultracentrifuge the purified preparation behaves as a polydisperse macromolecular substance.

The active material can be obtained from red cell stroma in an ether- and

chloroform-soluble form which, on further treatment, can be converted into chloroform-insoluble material. It is possible that the former represents more closely the virus receptor as it exists in the red cell.

The purified inhibitor is inactivated on incubation with the virus at 37°C. The nature of this effect is being investigated.

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BIBLIOGRAPHY

1. Bovarnick, M., and deBurgh, P. M., *Science*, 1947, **105**, 550.
2. Hirst, G. K., *Science*, 1941, **94**, 22.
3. Friedewald, W. F., Miller, E. S., and Whatley, L. R., *J. Exp. Med.*, 1947, **86**, 65.
4. Horsfall, F. L., Jr., and McCarty, M., *J. Exp. Med.*, 1947, **85**, 623.
5. Green, R. H., and Woolley, D. W., *J. Exp. Med.*, 1947, **86**, 55.
6. Salk, J. E., *J. Immunol.*, 1944, **49**, 87.
7. Jorpes, E., *Biochem. J.*, 1932, **26**, 1488.
8. Calvin, M., Evans, R. S., Behrendt, V., and Calvin, G., *Proc. Soc. Exp. Biol. and Med.*, 1946, **61**, 416.
9. McFarlane, A. S., *Nature*, 1942, **149**, 439.
10. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric Methods, Minneapolis, Burgess Publishing Co., 103.
11. Mejbaum, W., *Z. physiol. Chem.*, 1939, **258**, 117.
12. Dische, Z., *J. Biol. Chem.*, 1947, **167**, 189.
13. Folin, O., and Malmros, H., *J. Biol. Chem.*, 1929, **83**, 115.
14. Burnet, F. M., McCrea, J. F., and Stone, J. D., *Brit. J. Exp. Path.*, 1946, **27**, 228.
15. Takahashi, W. N., *Science*, 1946, **104**, 377.