# THE NATURE OF THE VIRUS RECEPTORS OF RED CELLS

## III. Partial Purification of the Virus Agglutination Inhibitor in Human Plasma

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The exposure of red cells to influenza virus results in the inactivation of their virus receptors in a manner which suggests that the process may be enzymatic in nature (1). Although many facts are in accord with the theory of enzymatic inactivation the final evidence must necessarily be the demonstration of a chemical change involved in receptor destruction. As a means to this end the isolation and identification of the cell receptor would be very helpful and it is along this line that the work reported below is directed.

One of the difficulties in isolating the cell receptor has been the lack of a suitable test for this substance after its separation from the cell. This problem was recently solved by Burnet and coworkers (2) and independently by Hirst (3) through the use of influenza virus heated at 56°C. Heated influenza virus retains its capacity for combining with receptors and for agglutinating red cells but loses its power of *inactivating* the receptor substance. As a result, virus altered by heat can be used for the detection of minute amounts of receptor-like substances in solution. The test is one of competition for combination with heated virus between the receptors in solution (the activity of which is being tested) and the receptors of red cells: virus which has combined with free receptors can no longer combine with receptors attached to red cells and hence can no longer agglutinate these cells. The receptor in solution is a virus hemagglutination inhibitor and will be subsequently referred to as VHI. VHI has been found in a wide variety of biological materials including fluid from pseudomucinous ovarian cysts (4), egg white (5), and extracts of many organs. In recent papers the VHI in rabbit serum was described (6, 3) and it was found that it had a number of properties in common with the receptors of red cells. Both serum VHI and cell receptors were destroyed by trypsin, periodate ions, cholera vibrio filtrate, and by native influenza virus and both were resistant to destruction by relatively high temperatures and oxidizing agents other than periodate. These similarities suggested that serum might be a suitable source of VHI for attempts at isolation and purification. This in turn led to the use of human plasma, which is rich in VHI, and had the further advantage that excellent mild methods of fractionation had already

been devised (7). While the separation of VHI from other plasma components is so far incomplete, the results have been of sufficient interest to warrant their description at this time.

### *Methods*

The Lee strain of influenza B virus has been used throughout. Suspensions of virus were prepared by the inoculation of the allantoic sac of 11-day-old embryonated eggs and the allantoic fluid was harvested after 2 days further incubation at 35.5°C. In order to remove the amorphous salts which precipitate from allantoic fluid, all preparations were dialyzed for several days against an M 0.11 phosphate buffer of pH 7.2. The suspensions were stored at 4°C.

The tests for VHI were all done in a manner similar to that used for the titration of antibody by agglutination inhibition (8, 3). Serial twofold dilutions of the VHI unknown were made in saline (0.5 cc. volume) and to each tube was added 0.5 cc. of saline containing 3 units of heated (56°C.) influenza virus. The mixtures stood for 1 hour to allow combination to take place. One cc. of 1.5 per cent fowl red cells was added and 75 minutes later the degree of agglutination was determined by means of a photoelectric densitometer (8). Three units of virus, in the absence of any inhibitor, agglutinated about 70 per cent of the red cells. The arbitrary end point of VHI activity was taken as half this value (35 per cent agglutination) since this fell in the zone where the degree of inhibition changed most rapidly with inhibitor dilution. End points which fell intermediate between two dilutions were calculated by interpolation in the same manner as end points for influenza antibody (8). One unit of VHI reduced the number of cells agglutinated by 3 units of virus from 70 to 35 per cent. The activity of various preparations was expressed as the number of units of inhibitor contained in 1 mg. of dried material. This expression is the same as the dilution factor of the unknown at the end point divided by 1000. Considerable variation occurred in the activity found in a given preparation when tested on different days. The reasons for this variation are not clear and comparisons of activity could be made only on materials tested at the same time.

## EXPERIMENTAL

In attacking the problem of identifying the influenza receptor substance of red cells, the indirect approach of purifying VHI from serum or plasma seemed to have certain advantages. As noted above and in a previous paper (6) the VHI in rabbit serum (or human plasma) and the red cell receptors had many properties in common, which suggested their essential similarity. Since the VHI content of human plasma is high and the essential component is in solution it was decided to concentrate efforts on this source of material. The initial attempts to purify VHI from plasma by drastic methods were not very successful and only the essential findings will be reported. Plasma fractions separated by milder techniques gave more promising results which are given in greater detail.

The titration of VHI in human plasma is complicated by the fact that all specimens contain influenza antibody, which also behaves as an inhibitor. This difficulty is, however, a minor one, since the amount of inhibition produced by antibody is relatively small compared to that due to VHI. Inhibition tests

with active virus on human plasma pools (mainly a measure of antibody) indicate that 10 per cent or less of the inhibition found in VHI tests was due to antibody. Of greater importance is the fact that antibody could be completely inactivated by heating without destroying the VHI.

*The Heat Stability of VHI in Plasma.*—One of the outstanding characteristics of VHI in plasma is its ability to withstand a temperature of 100°C. for at least several hours. Unheated plasma with a VHI content of 1000 units per cc. dropped in titer to 250 units per cc. after a short period of heating at 100°C., but prolonged heating at this temperature failed to lower the titer further. It seems most probable that the initial loss was due to activity being carried out of solution with coagulated protein to which it was adsorbed, since plasma fractions which did not contain coagulable protein lost no activity on boiling and in many instances increased in titer. This latter phenomenon will be described more fully below.

*Precipitation of VHI with Ammonium Sulfate.*—On addition of ammonium sulfate to human plasma (diluted 1:4 with distilled water) all VHI activity precipitated sharply between a concentration of 0.3 and 0.4 saturation. After plasma had been heated at 100°C., however, the VHI left in solution was only partially precipitated by salt concentrations as high as 0.6 saturation and the separation was not sharp at any level of salt concentration. The difference in precipitability with salt after heating could be due either to a change in the VHI or in other proteins to which VHI was adsorbed.

*The Effect of Concentrated Phenol on VHI.*—Previous work on the inactivation of VHI with periodate led to the tentative assumption that the active principle might be a mucin. Morgan and King found that blood group mucins were stable in phenol (9) while many other proteins were destroyed and they utilized this difference in separating blood group substances from crude sources by dissolving out extraneous compounds with 90 per cent phenol. An attempt was made to separate the VHI from plasma proteins by dissolving dried preparations in 90 per cent phenol. VHI activity was found in both the phenol-soluble and phenol-insoluble fractions, but only in small amounts. The low recovery rate may have been due to the inactivating effect of phenol on VHI since after exposure to this reagent for several days all traces of VHI disappeared. The VHI in plasma was very different from the VHI in O substance from pseudomucionous ovarian cysts which was quite stable in concentrated phenol.

*Fractionation of Plasma by Alcohol*[1].—Plasma has been separated into its major components by relatively mild methods developed at the Department of

[1] The products of plasma fractionation employed in this work were developed from blood collected by the American Red Cross, by the Department of Physical Chemistry, Harvard Medical School, Boston, under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

Physical Chemistry at Harvard Medical School (7). The fractions are precipitated by various concentrations of ethyl alcohol at different hydrogen ion concentrations at −5°C. The separation is accompanied by a minimum of protein denaturation. A complete series of plasma fractions was obtained through Dr. Geoffrey Edsall of the Massachusetts State Serum Laboratory. The various specimens were dissolved in physiological saline at a concentration of 1 per cent and were boiled at 100°C. for 30 minutes. The precipitated proteins were removed and the supernatants tested for VHI content. The results are given in Table I in terms of units of VHI per milligram of dried material. By far the greatest activity resided in the fraction designated IV-4 and a much smaller amount in fraction IV-1. By comparison the other fractions contained only traces. In the process of separation the IV-1 material

TABLE I

*Virus Inhibitory Activity of Fractions of Human Plasma*

| Plasma fraction | Units activity |
|---|---|
| I | 20 |
| II | <0.1 |
| III | 66 |
| IV-1 | 400 |
| IV-4 | 1,600 |
| V | 0.3 |

Fraction I was mainly fibrinogen, II gamma globulin, III gamma and beta globulins, IV-1 lipid-containing alpha and beta globulins, IV-4 non-lipid-containing alpha and beta globulins and albumin, and V serum albumin (7). All samples were dissolved in saline and boiled for 30 minutes at 1 per cent concentration. The coagulated protein was removed and inhibition tests were done with 3 units of heated Lee virus (56° C.)

was precipitated from solution at a pH of 5.2 and an alcohol concentration of 18 per cent. The IV-4 fraction was precipitated at pH 5.8 and an alcohol concentration of 40 per cent. The former fraction is rich in alpha globulins and lipids while the latter is mostly lipid-free alpha and beta globulins with some albumin (7).

The IV-4 fraction has been further separated into fractions IV-6 (mainly $alpha_2$ globulin), IV-7 (mainly $beta_1$ globulin), and IV-8 (serum albumin (10)). Samples of these fractions were kindly furnished by the Department of Physical Chemistry at Harvard Medical School. These fractions were boiled at 1 per cent concentration for 30 minutes and tested for their VHI content (Table II). The IV-8 fraction (serum albumin) contained almost no VHI. The IV-6 preparation had a lower degree of activity than the parent IV-4 fraction while the IV-7 fraction had the highest activity per unit weight, being about twice that of the IV-4 preparation. The main component of the IV-7

fraction (beta$_1$ globulin) has been crystallized (11) and a sample was made available through the Harvard laboratory. This crystalline substance had only one-third the activity of the IV-7 fraction from which it was obtained and hence the small amount of VHI present was probably a minor impurity.

*The Effect of Heating on the VHI Detectable in Plasma Fractions.*—A comparison of the VHI content of whole plasma and of the IV-4 fraction showed that nearly all the activity in plasma was recovered by the method whereby

TABLE II

*Virus Inhibitory Activity of Subfractions of the IV-4 Preparation*

| Fraction | Units activity per mg. |
|---|---|
| IV-4 | 450 |
| IV-6 | 340 |
| IV-7 | 1000 |
| IV-8 | <1 |
| $\beta_1$ globulin, crystallized 3 times | 315 |

Fractions IV-6, 7, and 8 were derived from the IV-4 fraction. IV-6 contains mainly alpha$_2$ globulins, the IV-7 mainly beta$_1$ globulin and IV-8 mainly albumin. The preparations were treated and tested after heating at 100°C. for 30 minutes.

TABLE III

| | Units activity per mg. |
|---|---|
| 1. Dried whole plasma | 8.7 |
| 2. IV-4 fraction in whole plasma | 110* |
| 3. IV-4 unheated | 100 |
| 4. IV-4 heated 100°C. | 910 |

See legend under Table I. The value for whole plasma boiled, though not done in this experiment would have been about one-fourth the value for unheated plasma.

* This is a calculated value for the IV-4 fraction contained in whole plasma using the average value of Cohn *et al.* (7) for the per cent of this fraction in whole dried plasma (7.75 per cent) applied to the activity of the latter. The similarity of values 2 and 3 indicates no loss of activity in fractionation.

IV-4 was separated (lines 2 and 3 of Table III). When the IV-4 fraction was heated at 100°C. for 30 minutes the VHI titer increased very greatly, often 8- to 16-fold over the unheated titer, and hence the yield of activity was many times greater than that found in the original plasma. The degree of change in VHI titer was found to depend on how much the plasma fraction was diluted at the time of heating (Table IV). When the IV-4 extract was boiled after dilution to 0.1 or 0.2 per cent, over 99 per cent of the VHI was destroyed. When the extract was boiled at a dilution of 3 per cent there was marked coagulation of protein but little change in activity. However, when the

extract was boiled at a dilution of 1.5 per cent there was a marked enhancement of VHI titer which in the example given was more than a 16-fold increase. The increase of titer with boiling was noted not only with the IV-4 but also with the IV-6, IV-7, and beta$_1$ preparations as well. The increase in activity on heating plasma fractions contrasts sharply with the behavior of VHI in whole plasma where a decrease of 75 per cent took place on boiling. In dilute

TABLE IV

*Effect of Concentration on Inhibitory Activity of the IV-4 Fraction Heated at 100°C.*

| Concentration of fraction when heated | Units activity per mg. |
|---|---|
| *per cent* | |
| Unheated | 110 |
| 3.0 | 130 |
| 1.5 | 2000 |
| 1.0 | 320 |
| 0.5 | 46 |
| 0.25 | 6.2 |
| 0.1 | <2 |
| 0.05 | <2 |

The extracts were heated in saline at pH 7.0 for 30 minutes at 100°C.

TABLE V

*Effect of Heating at Different Temperatures on the Inhibitory Activity of the IV-4 Fraction*

| Temperature of heating | Units activity per mg. |
|---|---|
| °C. | |
| Unheated | 43 |
| 60 | 22 |
| 70 | 38 |
| 80 | 130 |
| 90 | 200 |
| 100 | 320 |

The fraction was heated in 1.5 per cent concentration in phosphate buffer at pH 7.2 for 30 minutes.

solution the VHI of plasma fractions also differed from that in ovarian O substance, which was stable to boiling at a concentration of 0.1 per cent.

In the following experiment (Table V) the IV-4 fraction of plasma was heated at various temperatures up to 100°C. for 30 minutes and the effect on VHI titer tested. No significant change in titer was found after a temperature of 70°C. while after 80°C. there was a marked increase. The maximum effect was not reached until a temperature of 100°C. was employed.

A similar test to determine the optimum pH of activation with heating is shown in Table VI. In this test 1 per cent solutions of the IV-4 fraction at

different hydrogen ion concentrations were heated at 100°C. for 30 minutes. Heating the fraction at pH 5.0 and 5.5 resulted in a marked precipitation of protein and very little or no increase in activity. Heating at a pH of 7.9 or above resulted in complete loss of activity. The maximum activation was achieved by heating at a pH of 6.4.

The marked increase of VHI detectable after boiling plasma fractions suggests that the VHI in whole plasma may be combined with other proteins in such a way as to mask its presence. When coagulable proteins were present, as in whole plasma, their removal on heating was attended by some loss in adherent VHI. When no heat-coagulable proteins were present, as in the IV-4 and IV-7 fractions, the effect of heat may have been to dissociate VHI

TABLE VI

*Effect of Heating at Different Hydrogen Ion Concentrations on the Inhibitory Activity of the IV-4 Fraction*

| pH at which heated | Units activity per mg. |
|---|---|
| Unheated | 65 |
| 5.0 | 62 |
| 5.5 | 200 |
| 5.9 | 450 |
| 6.4 | 1,400 |
| 7.2 | 340 |
| 7.9 | <2 |
| 9.2 | <2 |

The fraction was heated at 100°C. for 30 minutes at the pH indicated. At pH 5.0–5.5 an acetate buffer was used, from 5.9 through 7.2 a phosphate buffer was used, and above 7.2 a borate buffer was used. The heated preparations were diluted in phosphate buffer at pH 7.2 for the inhibition test and the pH at the end point was at this level. Heavy precipitation occurred at pH 5.0–5.5 and increased turbidity at 5.9.

from the other proteins in such a way that a much greater proportion was left in active detectable form.

Elementary analysis of both the IV-4 and IV-7 fractions showed no detectable phosphorus, and the carbohydrate content, determined as reducing sugar after acid hydrolysis, was 2.97 and 3.50 per cent respectively. The active principle was precipitated from both these fractions by trichloroacetic acid and none of it was recovered from the precipitates even after dialysis. When the IV-4 fraction was shaken for several hours with a mixture of chloroform and butyl alcohol (12) a gel formed at the interface, and after the shaking out of all precipitable protein no detectable VHI was left in solution. The VHI in both the IV-4 and IV-7 fractions was readily destroyed by trypsin, sodium periodate, and unheated influenza virus just as occurred with VHI in normal rabbit serum (6, 3).

*The Content of VHI in Blood Group Substances.*—Burnet has reported the presence of large amounts of VHI in various blood group substances (2) and he attributed this activity to the blood group mucins themselves. Samples of blood group substances A, B, and O were obtained through the kindness of Dr. Elvin Kabat. These were obtained from human saliva, hog stomach, and from human stomach, and were of high specific serological activity and purity (13–15). All these preparations were found to contain less than 1 unit of VHI per mg., although simultaneous tests of plasma fractions gave values of 1000 units per mg. and higher. Blood group O substance from pseudomucinous ovarian cysts (obtained through the courtesy of Dr. W. T. J. Morgan) had a VHI content which was half that of the IV-7 plasma fraction. Unlike the VHI in plasma, the activity in this O substance was not destroyed by pepsin, trypsin, heating at 100°C. (concentration 0.1 per cent), or by 90 per cent phenol.

*Extraction of VHI from Red Cells.*—The resistance of VHI in plasma to boiling suggested the use of heat in extraction of receptors from red cells. Green and Woolley (16) have reported that the ghosts of fowl red cells yielded a virus agglutination inhibitor on boiling but they did not demonstrate that this inhibitor was destroyed by the virus. Fowl red cells were lysed with saponin and were washed until all hemoglobin was removed. The ghosts were suspended in saline (10 per cent concentration by volume), and were boiled for 1 hour. Samples were removed periodically and after sedimenting the cellular debris, the supernatant fluids were tested for VHI activity. The VHI content of the supernatant increased during the 1st hour of boiling after which the level declined.

The supernatant fluid from cells boiled for 1 hour was reduced in volume by drying in cellophane sacs. The excess salt was removed by dialysis and the active portion in solution was further concentrated by precipitation with 2 volumes of alcohol. This precipitate was dissolved in phosphate buffer and tested for VHI content. The inhibition curves were very similar in shape to those obtained with plasma fractions. The extraction of 1 gm. of wet red cells yielded an amount of activity equal to that found in 0.2 mg. of the IV-4 plasma fraction. The VHI from red cells was destroyed by periodate, trypsin, and unheated influenza virus. Further efforts in purifying this material are indicated.

## DISCUSSION

The suggestion that carbohydrate might be involved in the virus receptor complex was first made by Hirst (17) on the basis of receptor destruction by periodate. This effect does not suggest how great a portion of the molecule may be carbohydrate since Goebel, Olitsky, and Saenz (18) demonstrated the destruction by periodate of the biological activity of proteins in which carbohydrate was at most a minor constituent. Subsequently considerable evidence has accumulated suggesting that VHI or red cell receptors are mucoprotein in

nature. Much of this evidence (2), such as the occurrence of VHI in crude mucous secretions, the presence of mucinases in cholera vibrio filtrates, and the action of periodate is so tenuous that it adds almost no support to a firm conclusion regarding the nature of the agent and need not be discussed in detail.

Burnet and coworkers (19) found considerable VHI in purified blood group O substance prepared from pseudomucinous ovarian cysts and this has been cited by Anderson *et al.* (20) as direct evidence for the mucoprotein nature of VHI. The value of this finding as evidence depends largely on whether or not this substance is pure. In the light of available evidence a connection between blood group mucins and VHI is by no means certain. A number of observers have found that only blood group mucins prepared from ovarian cyst fluid contain significant amounts of VHI. At least one ovarian cyst fluid had a higher VHI titer even in the crude state than the most active blood group mucin of ovarian origin (19). Furthermore it has been shown that the destruction of VHI in blood group mucins does not involve the loss of any serological blood group activity (21). These facts plus the well known difficulty in purifying mucins strongly suggest that VHI is present in blood group mucins from ovarian cysts only as an impurity.

The most suggestive and direct evidence that red cell receptors are mucoproteins is provided by the work of de Burgh *et al.* (22) who isolated a preparation from human red cells with a carbohydrate content of 50 per cent, which inhibited the agglutination of heated virus to a very high titer (23) and was inactivated by normal influenza virus. McCrea (23) has obtained (in observations as yet unpublished) a potent VHI from ovarian cyst fluid which is stated to be a mucoprotein.

After the present study was completed, the results of McCrea (24) along similar lines became available. McCrea recovered 15 per cent of the total serum VHI in the serum mucoid fraction as prepared by Rimington (25). We obtained essentially the same result with this method except that the activity was completely precipitated by trichloroacetic acid. Serum mucoid is at best an ill-defined mixture and it is by no means certain that it contains only mucoids. Since the VHI in this fraction is precipitable by trichloroacetic acid and inactivated by shaking with chloroform and butyl alcohol, its behavior cannot be said to be typical of the other mucoids present.

The evidence presented in the present paper concerning the VHI of human plasma sheds little light on the chemical nature of this factor. On the basis of activity per unit of dry weight the IV-4 fraction was approximately 10 times and the heated IV-7 fraction 100 times as active as whole plasma. It is obvious that even the most active preparations are grossly impure and not comparable in activity to the extract of human red cells of de Burgh *et al.* (22). In some respects the VHI of plasma is very different from that found in blood group O substance of ovarian origin. The latter is very resistant to the action of 90 per cent phenol, of tryptic enzymes, and to boiling at high dilution, while plasma

VHI is not. This may be due to a chemical difference in VHI from the two sources or to the action of other substances in the preparations.

SUMMARY

A substance (VHI) exists in human plasma which inhibits the agglutination of red cells by influenza virus and is distinct from influenza antibody. When plasma is fractionated by alcohol in the cold the VHI comes out mainly with a mixture of lipid-free alpha and beta globulins (fraction IV-4). On further fractionation the activity comes out with a fraction consisting mainly of $beta_1$ globulin (fraction IV-7). Boiling fraction IV-4 or IV-7 after considerable dilution brings about a large increase in the amount of VHI, much more than can be detected in the original plasma. A similar VHI has been extracted from the ghosts of fowl red cells.

BIBLIOGRAPHY

1. Hirst, G. K., *J. Exp. Med.*, 1942, **76,** 195.
2. Burnet, F. M., *Lancet,* 1948, **1,** 7.
3. Hirst, G. K., *J. Exp. Med.*, 1948, **87,** 315.
4. Burnet, F. M., *Australian J. Sc.*, 1947, **10,** 21.
5. Lanni, F., and Beard, J. W., *Proc. Soc. Exp. Biol. and Med.*, 1948, **68,** 312.
6. Hirst, G. K., *J. Exp. Med.*, 1948, **87,** 301.
7. Cohn, E. J., Strong, L. E., Hughes, W. L., Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L., *J. Am. Chem. Soc.*, 1948, **68,** 459.
8. Hirst, G. K., and Pickels, E. G., *J. Immunol.*, 1942, **45,** 273.
9. Morgan, W. T. J., and King, H. K., *Biochem. J.*, 1943, **37,** 640.
10. Edsall, J. T. in Advances in Protein Chemistry, (M. L. Anson and J. T. Edsall, editors), New York, Academic Press, Inc., 1947, **3,** 383.
11. Personal communication.
12. Sevag, M. G., *Biochem. Z.*, 1934, **273,** 419.
13. Kabat, E. A., and Bezer, A. E., *J. Exp. Med.*, 1945, **82,** 207.
14. Kabat, E. A., Bendich, A., and Bezer, A. E., *J. Exp. Med.*, 1946, **83,** 477.
15. Bendich, A., Kabat, E. A., and Bezer, A. E., *J. Exp. Med.*, 1946, **83,** 485.
16. Green, R. H., and Woolley, D. W., *J. Exp. Med.*, 1947, **86,** 55.
17. The Rockefeller Foundation, International Health Division, Annual Report, New York, 1945, 50.
18. Goebel, W. F., Olitsky, P. K., and Saenz, A. C., *J. Exp. Med.*, 1948, **87,** 445.
19. Burnet, F. M., McCrea, J. F., and Anderson, S. G., *Nature*, 1947, **160,** 404.
20. Anderson, S. G., Burnet, F. M., Fazekas de St. Groth, S., McCrea, J. F., and Stone, J. D., *Australian J. Exp. Biol. and Med. Sc.*, 1948, **26,** 403.
21. Chu, C. M., *Nature,* 1948, **161,** 606.
22. de Burgh, P. M., Yu, P. C., Howe, C., and Bovarnick, M., *J. Exp. Med.*, 1948, **87,** 1.
23. Burnet, F. M., *Australian J. Exp. Biol and Med. Sc.*, 1948, **26,** 389.
24. McCrea, J. F., *Australian J. Exp. Biol. and Med. Sc.*, 1948, **26,** 355.
25. Rimington, C., *Biochem. J.*, 1940, **34,** 931.