PURIFICATION OF AN INFLUENZA VIRUS SUBSTRATE, AND DEMONSTRATION OF ITS COMPETITIVE ANTAGONISM TO APPLE PECTIN

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The ability of certain polysaccharides, notably apple pectin, to inhibit the hemagglutinating activity and the multiplication of influenza A virus has been described recently from this laboratory (1, 2). The discovery of this property of these polysaccharides depended on the recognition of the similarity of the virus acting on erythrocytes to an enzyme attacking its substrate. This view of the mode of virus hemagglutination has been proposed by Hirst (3) and subscribed to by Burnet *et al.* (4) as well as by many others. According to this postulate the virus contains an enzyme which attacks and destroys a specific substrate in the erythrocyte. While the virus is performing this function it is attached to the cell, and the result is agglutination of the latter. When the reaction has occurred, the substrate in the cell has been destroyed, and the virus is once more free in the solution and able to attack additional cells. The nature of this substrate is unknown and the purification of it will be the subject of the present report. In the previous work (1, 2) by utilizing existing evidence that the substrate might be carbohydrate in nature, we were led to try to inhibit the reaction by supplying an inhibitory structural analog of it to the virus-erythrocyte system (1, 2). Apple pectin as well as several other polysaccharides was found to effect this inhibition. If the working hypothesis be correct that such polysaccharide materials owed their antiviral action to competition for the virus with a related structure in the cell (the virus substrate or receptor), then it must follow that increased concentration of the substrate should antagonize a given concentration of the inhibitory polysaccharide. Viewed in this light, apple pectin would be inhibiting the action of influenza virus on the cell receptor just as malonate is known to counteract the action of succinic dehydrogenase on succinate. By adding malonate to the dehydrogenase-succinate system, the enzyme is inhibited, and one achieves a test for succinate because the addition of more of the latter allows the reaction to proceed. In the same way, if one were to add more of the virus receptor substance of the cell to the virus-erythrocyte system which has been inhibited by apple pectin, the inhibition should be relieved, and the normal reaction with the cell material (*i.e.* hemagglutination) should occur. More of the virus recep-

* With the technical assistance of R. Brown.

tor substance could be added either as more erythrocytes or preferably as extracts prepared from them. Therefore, the system of virus and red cell prevented from interacting by the presence of pectin should serve as a useful means of assaying for the receptor substance of the cell. By adding more of the latter, either as more cells or as extracts of the active principle of those cells, the inhibiting effect of the pectin should be erased. The end result in such an assay would be agglutination of the cells because when the inhibition is relieved by an increase in total concentration of the substrate in the system, combination and reaction of the virus with receptor molecules both in solution and in the cell should occur. While one cannot see the result of interaction of virus and dissolved substrate, that between virus and substrate in the cell appears as hemagglutination. If one could isolate the receptor substance from the cell, then it should be possible to demonstrate a reaction between it and the virus *in vitro* in the absence of any red blood cells or of inhibitor (apple pectin).

By the use of an assay procedure such as indicated above, a substance which antagonized the action of apple pectin has been extracted from erythrocytes of species susceptible to influenza virus. The antagonism between this material and apple pectin was competitive in nature over the range of concentration studied. Furthermore, the substance was purified to a considerable extent, and was found to be destroyed by highly purified influenza virus *in vitro*. Some of the properties of the substance, and the reaction of it with the virus have been studied.

Despite the success which has attended the use of the working hypothesis both in the interference with virus activity and in the study of substrates for the infecting agent, the validity of the picture of mechanism which it presents must remain open to question. At least, the postulate offers an explanation of the data thus far accumulated and has been fruitful of new knowledge.

After this manuscript was completed, a report by de Burgh *et al.* (5) on the purification of the influenza virus receptor appeared. These investigators used a quite different means of assay for the substance, which depended on the saturation of the virus with the substrate so that the former was no longer able to attack more substrate (*i.e.* red cells) in the time allowed for reaction. In this test, inhibition of hemagglutination was the criterion. It is quite possible that the mechanism of the inhibition of hemagglutination in this study was fundamentally different from that involving apple pectin. In this latter case, the virus may be hindered from attacking any of the substrate, while in the former it is gorged with the substrate so that it is unable to react with more in the time allotted for the test. The procedure used by de Burgh *et al.* may measure the same substance which will be described in the present work. In any event, both laboratories have arrived at similar conclusions on the nature of the influenza virus substrate in erythrocytes.

EXPERIMENTAL

Assay Method for the Demonstration of Substances Which Antagonize the Action of Pectin on Virus Hemagglutination.—In a series of test tubes (8 × 75 mm.) was placed 0.15 cc. of graded dilutions of a solution to be tested. This was followed by the addition of 0.1 cc. of a solution of apple pectin containing 5 mg. per cc.[1] Both of these solutions were prepared in a buffer solution made by dissolving 720 mg. of NaCl, 143 mg. Na_2HPO_4, and 242 mg. of NaH_2PO_4 in 100 cc. of water and adding 0.38 cc. of 1 M $CaCl_2$. The presence of $CaCl_2$ in the test solution was absolutely essential, because as will be shown shortly, calcium ions were required for the inhibition of viral hemagglutination by pectin. The buffer had a pH of 6.5. Following the mixing of the pectin and test solutions, each tube was treated first with 0.25 cc. of a 1 per cent suspension of washed chicken erythrocytes, and then with 0.25 cc. of a 1 to 160 dilution of allantoic fluid collected from embryonated chicken eggs which had been infected with the PR8 strain of influenza A virus. When the virus was propagated in the way previously described (2), this dilution of allantoic fluid contained approximately 4 times the amount of virus required to agglutinate erythrocytes in a system without pectin. The suspensions of virus and of erythrocytes were prepared in an 0.85 per cent NaCl solution. The assay tubes were shaken vigorously after each addition, and as little time as possible was allowed to elapse between additions. Furthermore, the order of addition just described for the various reagents was essential to the proper functioning of the test. After the tubes had been incubated at room temperature for 2 hours, the degree of hemagglutination was read and recorded in the manner described previously (2). If the solution under examination was active, complete agglutination occurred in its presence because the inhibition otherwise produced by the pectin had been entirely counteracted. The end point was taken as the greatest dilution which allowed complete agglutination of the cells. In each test, controls containing only erythrocytes, virus plus erythrocytes, and pectin plus virus plus erythrocytes were always included.

Effect of Calcium Ions on the Inhibition of Virus Hemagglutination Caused by Apple Pectin.—The demonstration of inhibition of hemagglutination by certain polysaccharides previously described (2) was done in a test system of 0.85 per cent NaCl. In such a system, inhibition was demonstrated readily. However, if a buffer such as phosphate or phosphate plus citrate was used instead, apple pectin caused no inhibition of virus action. When small amounts of calcium ion were added to a phosphate buffer at pH 6.5, the action of pectin could once more be demonstrated. From these facts it would seem that it is the calcium salt of pectin, and not just the polysaccharide itself, which is the inhibiting agent. All attempts to demonstrate participation of calcium ions in the reaction between virus and erythrocyte have failed. For example, the hemagglutinating titer of a virus suspension was not influenced by phosphate or by citrate.

Demonstration in Hemolyzed Erythrocytes of a Substance Antagonistic to the Action of Pectin.—Erythrocytes from chickens or human beings were washed 4 times with 0.85 per cent NaCl solution, and the packed cells were suspended in

[1] It was essential to prepare solutions of apple pectin by the manner described previously (2). Solutions made in a Waring Blendor in the cold were ineffective.

100 times their volume of ice cold water. As soon as the cells were hemolyzed, enough NaCl was added to give a concentration of 0.85 per cent, and the mixture was centrifuged in the cold. The clear, red supernatant liquid was found to be quite active in reversing the effect of apple pectin on virus hemagglutination when tested in the manner described above. Representative data are shown in Table I. It was necessary to conduct all manipulations in the cold, and as rapidly as possible because of the labile nature of the antagonistic agent.

High concentrations of the antagonistic agent in hemolysates showed no activity in the regular assay system containing pectin, but dilution of these preparations allowed demonstration of their potency. These facts can be seen

TABLE I

Antagonistic Effect of an Hemolysate of Chicken Erythrocytes on the Inhibitory Action of Apple Pectin towards Influenza Virus Hemagglutination

Hemolysate		Apple pectin	Phosphate buffer	RBC suspension	Virus suspension	Hemagglutination*
Dilution	Cc.					
		cc.	*cc.*	*cc.*	*cc.*	
0	0	0	0.50	0.25	0	0
0	0	0	0.25	0.25	0.25	c
0	0	0.10	0.15	0.25	0.25	t
0	0.15	0.10	0	0.25	0.25	t
1:10	0.15	0.10	0	0.25	0.25	c
1:1000	0.15	0.10	0	0.25	0.25	c
1:10,000	0.15	0.10	0	0.25	0.25	p
1:100,000	0.15	0.10	0	0.25	0.25	p
1:10	0.15	0	0.35	0.25	0	0

* c = complete, t = trace, p = partial as described previously (2).

in the data of Table I. Perhaps when sufficient concentration of the pectin antagonist was present *in solution*, practically all the virus combined with it before an erythrocyte was encountered by the virus particles, and therefore only an insignificant amount of the latter united with the receptor *in the cells*.

In making extracts of erythrocytes which would be capable of antagonizing the action of pectin, it was necessary always to test the preparations for ability to agglutinate red cells in the absence of virus. Especially from stromata one could readily obtain fat-soluble substances which by themselves agglutinated red cells. The presence of such materials in solutions being examined for anti-pectin action would lead to false conclusions. None of the fractions described in this investigation caused agglutination in the absence of the virus. During examination of stromata for possible antagonistic agents it was observed that lipids which may be extracted from them with ether, and which are hemagglutinins, could be readily removed by adsorption on ether-extracted stromata.

Presence of the Pectin Antagonist in Normal Rabbit Serum.—Recent investigations (6) have indicated that the inhibitor of virus hemagglutination which is found in normal sera is related to, or is identical with, the receptor in red cells. If this be true, and if the thesis of the present paper is correct, then sera should be a good source of the pectin antagonist. Therefore, normal rabbit serum was assayed in the test system described above and the results are shown in Table II. Because such serum will inhibit virus hemagglutination without any pectin being present, a parallel titration was conducted to measure the extent of this inhibition. The data in Table II will show that much smaller quantities of

TABLE II

Antagonistic Effect of Normal Rabbit Serum on the Inhibition by Apple Pectin of Virus Hemagglutination and the Effect of the Same Serum on Virus Hemagglutination without Pectin

Rabbit serum		Apple pectin	Phosphate buffer	RBC suspension	Virus suspension	Hemagglutination
Dilution	Cc.					
		cc.	*cc.*	*cc.*	*cc.*	
0	0	0	0.50	0.25	0	0
0	0	0	0.25	0.25	0.25	c
0	0	0.10	0.15	0.25	0.25	p
1:10	0.15	0.10	0	0.25	0.25	t
1:100	0.15	0.10	0	0.25	0.25	0
1:1000	0.15	0.10	0	0.25	0.25	c
1:10,000	0.15	0.10	0	0.25	0.25	c
1:100,000	0.15	0.10	0	0.25	0.25	p
1:10	0.15	0	0.10	0.25	0.25	0
1:100	0.15	0	0.10	0.25	0.25	p
1:1000	0.15	0	0.10	0.25	0.25	c
1:10,000	0.15	0	0.10	0.25	0.25	c

serum were effective in the system containing pectin than in the one without it. This fact might serve as evidence for the statement in the last paragraph of the introduction.

The Necessity for Fresh Viable Virus for Demonstration of the Phenomenon.—In order to demonstrate the antagonism of hemolysates or of normal serum to the antiviral action of apple pectin, relatively fresh virus or a preparation which had been stored in such a manner as to maintain infectivity was essential. Virus-containing allantoic fluid held under conditions in which infectivity was diminished could not be used. For example, virus-containing allantoic fluid which had been kept for many months in a chest cooled with solid carbon dioxide had lost only 50 per cent of its activity as a hemagglutinin, but was approximately 1/100,000 as infective for embryonated eggs as it had been when harvested. This preparation of virus was tested in the assay described in the

previous sections, and no antagonism between pectin and the active hemolysate, or between pectin and the rabbit serum, could be detected. Nevertheless, with this aged virus preparation the action of apple pectin in causing inhibition of hemagglutination still could be demonstrated. When this virus was cultivated by one passage in the allantoic sacs of chicken embryos, the infective virus so obtained was again capable of use in the assays for antagonistic effect. Thus it appeared that the infective agent was necessary for demonstration of the phenomenon. From the experiments of Francis (7) and of Hirst (6) the need for unaltered virus for the elucidation of the enzyme-like action of influenza virus on erythrocytes is clear.

Competitive Nature of the Antagonism between Apple Pectin and the Substance from Erythrocytes.—Preparations of the antagonistic agent obtained as described above were titered in a series of dilutions of apple pectin. With each concentration of the pectin, the dilution of antagonistic substance which just allowed complete agglutination by the virus was determined. The data are summarized in Table III, where it can be seen that when the concentration of pectin was doubled, the amount of antagonistic agent required to overcome it was correspondingly increased (within the range of experimental error of the test). In other words, the antagonism between apple pectin and the red cell extract was competitive in nature, over the range of concentration studied.

TABLE III

Amounts of Hemolysate of Chicken RBC Required to Antagonize Varying Concentrations of Apple Pectin

Dilution of hemolysate	Extent of hemagglutination in the presence of apple pectin			
	2.5 mg. per tube	1 mg. per tube	0.5 mg. per tube	0.25 mg. per tube
No hemolysate	t	p	p	p
1:400	t	c	c	c
1:800	t	p	c	c
1:1600	t	p	c	c
1:3200	t	p	p	c
1:6400	t	p	p	p

Recovery of Pectin after Mixing with Antagonistic Agent.—Because the effect of the active agent in the erythrocyte extracts may have been due to an enzyme or other substance which destroyed pectin, the following experiment was done to learn whether the pectin could be regained after mixing with the extract. One cc. of apple pectin solution (5 mg. per cc.) was mixed with 1.5 cc. of hemolysate from chicken erythrocytes prepared as described above. The mixture was held at 25° for an hour, and then heated to 100° for 5 minutes, cooled, and centrifuged. Assays showed that while the original mixture of pectin and cell extract would not inhibit agglutination of red cells by influenza virus, that which had

been heated to destroy the antagonistic agent was fully potent in this regard. In other words, the antiviral action of pectin was nullified by the antagonistic agent, and was recovered from the mixture by destroying this agent with heat. The same result was accomplished by incubating the mixture at 37° for 24 hours. This procedure likewise was found to destroy the potency of the hemolysate.

Effect of Calcium Ions on the Activity of the Antagonistic Agent from Erythrocytes.—Because a substance such as citrate, which binds calcium ions, would overcome the action of pectin as described in an earlier section, it must be determined whether the antagonistic agent from erythrocytes acted merely by removing calcium ions from the system. When potent hemolysates of chicken erythrocytes were made 0.01 M with respect to calcium chloride, their activity was not influenced. However, when purified preparations were made 0.02 M with respect to calcium chloride, practically all potency was destroyed. These higher concentrations of calcium produced a slight precipitate in the purified preparations. One may conclude tentatively that the antagonistic agent was precipitated by sufficient amounts of calcium ions. Nevertheless, this effect of calcium ions raised a serious question as to whether the erythrocyte substance was merely able to remove these ions from the system just as phosphate or citrate did. However, since the purified virus was shown to react with the pectin antagonist from the cells (see below), the likelihood of this latter being the virus substrate of the cell was increased.

Relative Concentration of the Antagonistic Agent in the Erythrocytes of Various Species.—Hemolysates of washed erythrocytes of various animal species were prepared in the manner described above, and these were titered in the presence of the standard amount of pectin. The results shown in Table IV demonstrate that some of the agent occurred in all species examined. Human cells were by far the richest source.

TABLE IV

Relative Concentration of Apple Pectin Antagonist in Hemolysates of Erythrocytes of Various Species

Species	End point dilution of hemolysate	Agglutinability of RBC by influenza virus
Chicken	1:1000	Agglutinable
Human	1:1,000,000	Agglutinable
Mouse	1:10,000	Agglutinable
Beef	1:10,000	Not agglutinable
Sheep	1:100	Not agglutinable*

* Some individual sheep give cells which are agglutinable, but these were not encountered in the present work.

Destruction of the Antagonistic Agent by Incubation with Influenza Virus.—Influenza A virus was found to destroy the erythrocyte substance. Thus when

a mixture of 1 cc. of potent extract of chicken erythrocytes and 0.1 cc. of undiluted virus-infected allantoic fluid was incubated at 25°C. for 3 hours, and then diluted, assays showed that about 75 per cent of the antipectin activity had been destroyed. When a suspension of 22 gamma of highly purified influenza virus[2] was used in place of the infected allantoic fluid, 90 per cent of the activity was lost. Controls in which the erythrocyte extract was mixed with 0.85 per cent NaCl instead of the virus showed no loss. However, this was not uniformly true, for with some preparations of the erythrocyte extract, activity was largely destroyed in 3 hours at room temperature in the absence of virus. This difficulty was overcome by the finding that stromata of red cells stabilized the extract. Thus, when chicken erythrocytes were hemolyzed with water, but the stromata were allowed to remain suspended in the hemolysate, activity of the latter against pectin was retained after storage at 25° overnight. Before testing such a preparation it was freed of cell debris by centrifugation. By taking advantage of this fact, it could be regularly demonstrated that incubation of the suspension of stromata in hemolysate with either crude or highly purified virus resulted in destruction of about 90 per cent of the agent antagonistic to pectin.

The antagonistic agent could also be destroyed by incubation of intact erythrocytes with the virus. Thus, when 5 cc. of a 1 per cent suspension of washed chicken erythrocytes in 0.85 per cent NaCl solution was exposed to 0.1 cc. undiluted virus-infected allantoic fluid overnight, and the cells were collected by centrifugation and then washed thoroughly, an hemolysate prepared from them in the usual fashion had only one-tenth the potency of one prepared from cells not exposed to virus.

Purification of the Active Substance in Erythrocytes.—Six cc. of human erythrocytes which had been washed 4 times with 0.85 per cent NaCl solution was suspended in 60 cc. of water at 0°. 510 mg. of NaCl was added and the mixture was centrifuged at 0°, and the stromata were washed once with 10 cc. of cold NaCl solution. The extract was immediately stirred and treated with 180 cc. of cold alcohol. The mixture which resulted was kept at 0° for 30 minutes, and then the precipitate was removed by centrifugation and the supernatant liquid discarded. The precipitate was extracted twice in the cold with a total volume of 35 cc. of cold 0.85 per cent NaCl solution, and the active extract so obtained was shaken with chloroform and centrifuged. The aqueous phase was separated from the gel and the chloroform layer, and the shaking with chloroform was repeated until no more precipitate formed. The colorless aqueous solution was concentrated slightly under reduced pressure to remove chloro-

[2] The preparation of highly purified influenza A virus was very kindly supplied by Dr. C. A. Knight. It had been prepared by adsorption on and elution from erythrocytes and further purification by differential centrifugation as described by Knight (8).

form, and was then treated in the cold with 3 volumes of cold alcohol. A white precipitate was formed which was collected by centrifugation, washed, and dried; yield 40 mg. The product dissolved with great difficulty after it had been dried, and for this reason it was best to dissolve the final alcohol precipitate in 0.85 per cent NaCl solution, or in water while it was still moist. In this way products were obtained which were active at 0.00004 gamma. In other words, the action of 0.75 mg. of apple pectin in causing inhibition of virus hemagglutination was overcome by 0.00004 gamma of this substance. The major part of the activity of the crude hemolysate was recovered in the purified material. The relative inactivity of apple pectin as a virus inhibitor may be seen readily from the minute amount of the erythrocyte substance which antagonized it. The inhibition index (9) was $\frac{750}{0.00004} = 19{,}000{,}000$.

Some Properties of the Active Substance.—The active agent as obtained above was a white powder which appeared as small scales under the microscope. It was a water-soluble, non-dialyzable material insoluble in all organic solvents tested. It was precipitated by saturation of an aqueous solution with ammonium sulfate, but not by half saturation. Solutions of the substance gave a Molisch test for carbohydrate. Its activity was not influenced by exposure for 1 hour at 0° to 1 N NaOH or 1 N HCl. When aqueous solutions were heated to boiling a precipitate formed, and all activity was destroyed. One property which was outstanding in this investigation was the unstable character of the substance in aqueous solution. Storage of such solutions for a week at 4° resulted in complete inactivation. At room temperature, it was much less stable.

Changes in Viscosity during Interaction of Virus and Active Agent.—When influenza A virus was mixed with the substance isolated from human erythrocytes, not only was the latter destroyed, but a decrease in viscosity resulted. Determinations were made with 0.1 per cent solutions of the purified substance in 0.85 per cent NaCl. Five cc. of solution was placed in an Ostwald viscosimeter at 30.0°C. Two-tenths cc. of a suspension of highly purified influenza virus[2] (225 gamma per cc.) in the phosphate buffer described earlier in this paper was added and readings were taken at 5 minute intervals. From the data plotted in Fig. 1, it can be seen that viscosity fell as the reaction occurred. A preliminary rise in viscosity was noted uniformly shortly after mixing of the reactants. The magnitude of the changes was not great, but the effects were readily reproducible. Controls without virus were run at the same time, and showed no change in viscosity.

Purification of a Second Substance in Erythrocytes Which Counteracted the Action of Pectin, but Which Was Not Attacked by the Virus.—When washed chicken erythrocytes were suspended in 10 times their volume of glycine

buffer,[3] and the mixture adjusted to pH 9.0 and stored at 0° for 15 minutes, a substance was extracted which counteracted the inhibitory action of pectin. To obtain this material in purified form, the cells in the buffer at pH 9 were removed by centrifugation, and the pale yellow extract was adjusted to pH 6.5, and shaken with chloroform until no more precipitate formed. The colorless aqueous extract was then dialyzed in the cold until free of salts.

This material was extractable from erythrocytes only over a narrow range of pH. Very little was obtainable at pH 9.4 and none at a pH below 8.0. Maximal yields were found at pH 9.0. Crude preparations were rather unstable because they contained an enzyme which slowly inactivated the antipectin

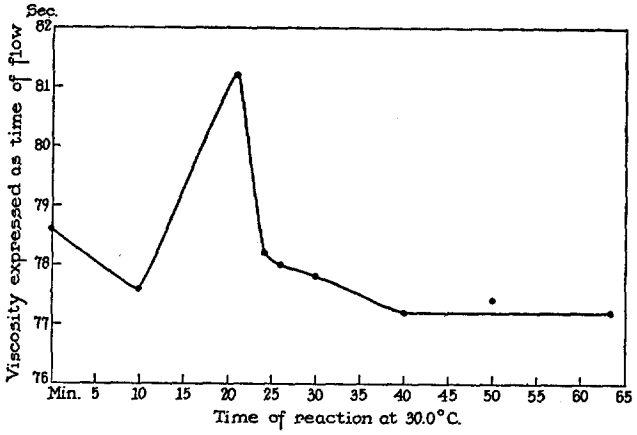

FIG. 1. Changes in viscosity during reaction of influenza virus and purified erythrocyte substance.

substance even at 4°, but this factor was removed by the precipitation with chloroform, so that purified preparations were stable for several months at 4°. Crude extracts obtained at pH 9.0 were less active in antagonizing the action of pectin on the virus-erythrocyte system than were extracts prepared by hemolysis as described earlier. Thus, hemolysates of chicken cells were active to a dilution of 1 to 10,000, while the pH 9 extract was only effective to a dilution of 1 to 30.[4] Furthermore, the substance in the pH 9 extract was stable to boiling, and to storage, whereas the active agent in the hemolysate was not.

A large number of species were investigated for the occurrence of the pH 9-extractable substance, and it was found in the erythrocytes of chicken, human being, beef, sheep, goat, and pig, and each of these gave preparations of

[3] The buffer was prepared by dissolving 3.8 gm. glycine and 7.5 gm. NaCl in 990 cc. H_2O and adding 10 cc. 1 N NaOH.

[4] Comparison in both cases was made on the basis of 1 cc. of erythrocytes per 10 cc. of final extract.

about the same potency. None was found in the red cells of rabbit, mouse, or rat. This lack of correlation of its occurrence with ability of the virus to cause agglutination of the respective red cells suggested that it was not the substance in erythrocytes with which the virus reacted.

Further conclusive evidence on this point was at hand when it was found that the virus would not inactivate the material. After extraction at pH 9, chicken erythrocytes were still intact, and even after repeated extraction they were readily agglutinated by the virus. Five successive treatments of the intact cells with the virus did not reduce the yield of the active material which could be extracted from them at pH 9.

The action of this substance could be completely abolished by the addition of small amounts of calcium chloride, and potency could be restored by dialysis. It would therefore seem that this was a non-specific agent which owed its activity to the ability to bind calcium ions.

DISCUSSION

The data obtained in this investigation are compatible with the hypothesis that the substance purified from hemolysates of human erythrocytes is a material in the cell with which the influenza virus reacts. Not only does the substance compete with pectin in virus hemagglutination, but also it is destroyed by the virus *in vitro*. The competitive nature of the relationship between pectin and this substance from susceptible cells adds some credence to the original working hypothesis (1, 2) that apple pectin and certain other polysaccharides cause inhibition of hemagglutination and of multiplication of the virus by retarding an enzymic attack by the virus on a structurally similar polysaccharide in the cell. However, one cannot regard the hypothesis as proven fact, especially since calcium ions are intimately concerned with the inhibition caused by pectin as well as with the action of the erythrocyte substance in antagonizing it. For the moment it seems probable that calcium ions inactivate the erythrocyte substance by precipitating it.

Although the working hypothesis which pictures the inhibitory action of pectin as being due to competition with a structurally similar substance in the cell has led to the facts described in this paper, it may be possible to fit these facts to some other view of mechanism. Some investigators have favored the postulate that the antiviral action of polysaccharides should be attributed to a coating or varnishing of the cell surface by these agents. Others (10) have considered that the polysaccharides may interfere in undetermined manner with the *internal* metabolic reactions of the cell in such a way as to inhibit the activities of the virus.

In integrating the experimental findings on inhibition of influenza virus and of bacteriophage (11) with polysaccharides, one sees that the process of invasion of the cell is probably the point of action. Especially with citrus pectin acting

on bacteriophage, the polysaccharide seems to interfere with penetration of the virus into the interior of the cell. The same may be true for apple pectin acting against the influenza virus. The decrease in viscosity which this virus causes in the material isolated from erythrocytes might therefore be of importance during invasion.

The substance purified from erythrocytes appears to be of polysaccharide nature. At least it is a non-dialyzable material, precipitable by alcohol, and not precipitated by chloroform, a reagent which readily removes most proteins from solution. Since the preparations contained some nitrogen, the active agent may be a conjugated polysaccharide in which some nitrogenous component occurs. The purified material gives qualitative tests for carbohydrate, but since the most active preparation is probably not a pure substance, final decision on its chemical nature must await the development of adequate means of purifying polysaccharides.

SUMMARY

A substance was demonstrated in erythrocytes which antagonized the inhibiting effect of apple pectin on influenza virus hemagglutination. This substance was purified and found to be a water-soluble material, rather labile, and with some properties which suggested that it contained a polysaccharide. It was destroyed *in vitro* by highly purified preparations of the virus. It occurred in greatest amount in human erythrocytes and to a lesser extent in the red cells of species not susceptible to the virus. It was also found in normal rabbit serum.

Calcium ions were found to be essential to the action of apple pectin in causing inhibition of virus hemagglutination. A second substance was purified from an alkaline extract of erythrocytes, and shown likewise to have an effect antagonistic to that of pectin. However, this latter material was not destroyed by the virus, and seemed to owe its effect to the binding of calcium ions.

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