# THE INFLUENZA VIRUS ADSORBING CAPACITY OF THE VASCULAR ENDOTHELIUM OF VARIOUS MAMMALS

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Haemagglutination caused by influenza and related viruses is inhibited in a significant degree by tissue extracts and body fluids. As regards the details of this problem, we refer to the works by *Burnet* [1, 2, 3] as well as to studies published by *Anderson* [4], *Bieling* and *Oelrichs* [5]. Not only erythrocytes, but also other tissues possess virus adsorbing capacity. Mitochondria isolated from hepatic cells bind the virus of influenza [6]. Of a virus introduced into the excised lung of mammals, considerable amounts are adsorbed by the pulmonary epithelium [7, 8, 9]. This latter phenomenon is equally observable in animal species not susceptible to the virus [9].

We have, however, been unable to find data in the literature to decide whether the vascular endothelium is capable of adsorbing virus. It has been thought that the problem could adequately be studied in organs removed from the body and perfused with influenza virus material. The experimental procedure adopted for this purpose was essentially the following. Under anaesthesia, the vena portae of the animal was cannulated. Subsequently the liver, carefully perfused with saline, was removed from the abdominal cavity and a suspension of virus of known titre was circulated in the blood vessels. The observations made in the course of these investigations are the subject matter of the present paper.

## *Material and Methods*

*Virus material.* Allantoic fluid from infected chick embryos was used. In cases where virus was titrated by the haemagglutination method only, fresh, not more than two weeks old, allantoic fluid stored in the frozen state at — 20°C was employed. In cases where the reduction of infectivity titre was also studied in order to follow the changes in the degree of virus adsorption, virus material harvested on the day of the experiment was used.

*Titration of the virus.* The method employed for determining HA titres has been described [9]. The fluid dripping off the hepatic vein was titrated for haemagglutination, both undiluted and in a serial dilution beginning 1 : 2. The original samples and their dilutions were mixed with an equal volume (0,1 ml) of a chicken erythrocyte suspension. Agglutination was read after 45 minutes. If no agglutination could be detected in the first tube, the titre was taken as «< 2».

Infectivity was titrated according to *Horváth* [10], in semi-micro tissue cultures made of chorio-allantoic membrane suspensions. As to the details of the procedure, the paper by

*Horváth* should be consulted. Five parallel semi-micro cultures were made of each virus dilution and after 72 hours' propagation the rate of multiplication was estimated by the haemagglutination method. The method allowed to estimate the infectivity titre within a margin of error of $\pm$ 0,3 log units.

*Removal and perfusion of liver.* With due regard to the experience of *Jancsó* [11], the following technique was employed in making the liver preparation. Young guinea pigs (250 to 300 g), adult rats (280 to 350 g), as well as old albino mice (25 to 30 g) were used. The animals were laparatomized under ether anaesthesia, the portal vein was gently compressed and through an incision made in its wall a glass cannula of suitable size, filled with saline, was introduced in the direction of the liver and held in place by ligation. Without delay the liver was perfused with saline to remove blood. Then the thorax was entered through the diaphragm, the inferior vena cava severed above the diaphragm and the animal was bled to death. The diaphragm was detached from the thoracal wall, the liver gently raised, the peritoneal parts and ligaments were cut and the liver so freed was removed from the abdomen. Meanwhile, saline with physiological perfusion was continued. The liver was placed on an inclined glass plate, $9 \times 12$ cm in size, with one corner as the lowest point. The fluid oozing out of the liver flowed toward this point, where it dripped off. The rate of flow could satisfactorily be estimated by counting the drops. The glass cannula inserted into the liver was attached to a glass coil in a water bath by means of a thin rubber tube. The temperature of the fluid passing through the coil could thus be regulated. The coil, whose internal diameter was 4 mm, ended in a small glass container, which formed the highest part of the system. The fluid poured into the container, after passing through the coolable coil and the liver, left through the hepatic vein, collected in small drops on the glass plate and dripped into a volumetric cylinder. The fluid collected in the cylinder was at intervals re-introduced into the glass container, in order to maintain a constant flow of the virus-bearing fluid. The total capacity of the coil and the cannula was 21 ml. For mouse livers the volume of the system was adequately reduced by means of shortening the tubing. The liver of guinea pigs and rats used in the experiment took up an average of 5 ml of fluid, while mouse liver could take up about 2 ml. These values were duly considered and were added to the volume of the tubing.

Prior to circulating the virus-bearing fluid, the coil was kept in melting ice and the liver was perfused with ice-cold dilute formaldehyde and saline for 30 minutes. As a result, the liver was considerably chilled and the thermometer inserted between its lobes showed temperatures of 8° to 9° C.

When the level of the last portion of the washing fluid reached the outlet of the glass container, the virus-bearing fluid was immediately poured into the container. In this way errors due to dilution of virus could be practically eliminated. Cooling was maintained while circulating the virus. Immediately after the virus was measured into the container we began to measure the volume of the fluid dripping out of the liver until 26 ml (or, with mouse livers, a proportionate volume) had collected, which was then discarded. After that moment the fluid dripping out of the liver was considered to be the virus material. The first 4 ml of this fluid collected in the volumetric cylinder was set aside as sample 1. This sampling marked the beginning of the experimental period. The collected portions, in the order of collection, were successively re-introduced into the container at the very moment it became empty. Precautions were taken to prevent bubbles of air from entering the system. By this simple method a steady flow of virus material could be maintained in the system. The livers of guinea pigs and rats were perfused with 40 ml, the mouse livers with about 20 ml of virus-containing fluid. It took about 10 minutes for the 40 ml of fluid to pass through the liver.

*Control of liver preparations.* According to *Jancsó* [11], the liver perfused with metal colloid sols filters out metal particles from the fluid. This is, however, not to be ascribed to Kupffer cell function, but is the result of the so-called »ultrafiltrative action« of the liver. In the presence of hydrophil colloids (serum protein, gelatin) the activity of Kupffer cells is markedly increased while in the absence of proteins the adsorbing capacity of the hepatic capillaries becomes very significant.

When a carefully washed liver preparation was perfused with India ink diluted with saline, the fluid dripping out of the hepatic vein was practically colourless. Microscopic examination of the liver revealed that the capillaries were obstructed by a very large number of India ink granules, but there were no such granules detectable in the Kupffer cells. If a mixture of India ink and bovine serum was used for perfusion, the outflowing fluid did not differ in colour from the original. In such cases histological examination showed no precipitated granules of India ink, but such granules were stored in the Kupffer cells.

Liver preparations treated with formaldehyde bound the introduced virus gradually, while untreated livers bound all the virus contained in the perfusion fluid on the first occasion already. These phenomena seem to indicate that on treatment with formaldehyde the non-specific adsorbing capacity of hepatic capillaries becomes either abolished or very greatly reduced.

For this reason the livers used were perfused with formaldehyde and washed thoroughly. Adsorption of virus was considered characteristic only if on rewarming the organ at a later point of time the virus could partly be eluted.

On completion of the experiment, the liver was perfused with 5 ml of dilute methylene blue in physiological saline, in order to determine whether all the lobes participated in the perfusion, indicating that the cannula was introduced properly.

## *Results*

The liver preparations made as described above were perfused with influenza PR8, Lee, Swine, as well as with Newcastle virus. The experiments were carried out as follows.

The liver was perfused with chilled physiological saline and completely freed from blood. Then it was perfused with 100 ml cold physiological saline containing 0,5 per cent formaldehyde which was then removed by washing with cold saline for 20 to 25 minutes. In the liver so treated allantoic fluid diluted with saline at a ratio of 1 to 4 was circulated. Circulation of the allantoic fluid was continued for 60 minutes with samples taken at the beginning of the experiment, and then in intervals of 15 minutes. The samples were tested for haemagglutination. The allantoic fluid was then allowed to flow out of the liver, which was then perfused with 40 ml of chilled physiological saline for 30 minutes. Samples for HA testing were taken at 15 and 30 minutes.

After this, the rubber tube connecting the cannula to the coil was clamped, the liver and the cannula inserted into it were detached from the system and, covered with a piece of gauze soaked in physiological saline, they were placed for 30 minutes into an incubator of 37°C. At the end of this period the liver was perfused with 2 ml of body-warm physiological saline injected into the rubber tube on the cannula by means of a syringe equipped with a fine needle. The fluid dripping out of the liver was tested for haemagglutination. The entire procedure was then repeated and sampling after half an hour obtained. All this was made in order to find out whether the virus bound by the chilled liver would be released at body temperature. The results are summarized in Table I.

As shown in Table I, the 9 experiments performed with various viruses on the livers of guinea pigs, rats and mice, respectively, yielded unequivocal results. The virus became gradually bound in the formaldehyde-treated, washed and chilled livers and by the end of this stage of the experiment HA tests failed to demonstrate the presence of virus. The chilled liver retains all, or at least most, of the virus bound, as was shown by the fact that subsequent perfusion with chilled water yielded a washing fluid which contained no such amounts of virus as are detectable by haemagglutination. Illustrative of the partly reversible nature of virus adsorption is the HA titre of samples taken after the liver had been warmed up. Such samples yielded varying agglutination titres in the individual experiments.

TABLE I

*Summary of Liver Perfusion Experiments in Different Animals*

| Species of exp. animal and titre of virus used | Stage of experiment | Time of sampling (min)* | HA titre of sample** | | | |
|---|---|---|---|---|---|---|
| | | | PR8 | Lee | Swine | NDV |
| *Rat* | | 0 | 16 | 48 | 48 | 128 |
| PR8 160 | | 15 | 8 | 24 | 48 | 64 |
| Lee 160 | Perfusion with virus | 30 | < 2 | 16 | 8 | 32 |
| Swine 160 | | 45 | < 2 | 2 | 2 | 32 |
| NDV 160 | | 60 | — | — | < 2 | 16 |
| | Perfusion with saline | 15 | < 2 | < 2 | < 2 | < 2 |
| | | 30 | < 2 | < 2 | < 2 | < 2 |
| | Elution in incubator | 30 | 12 | 24 | 8 | 8 |
| | | 60 | 12 | 32 | 8 | 8 |
| *Guinea pig* | | 0 | 16 | 16 | | 48 |
| PR8 256 | | 15 | 8 | 8 | | 24 |
| Lee 128 | Perfusion with virus | 30 | 8 | 4 | | 24 |
| Swine 192 | | 45 | 4 | < 2 | | 12 |
| | | 60 | — | — | | 4 |
| | Perfusion with saline | 15 | < 2 | < 2 | | < 2 |
| | | 30 | < 2 | < 2 | | < 2 |
| | Elution in incubator | 30 | 128 | 128 | | 16 |
| | | 60 | 48 | 128 | | 16 |
| *Mouse* | | 0 | 24 | | | 32 |
| PR8 96 | | 15 | 12 | | | 16 |
| NDV 128 | Perfusion with virus | 30 | 12 | | | 16 |
| | | 45 | 4 | | | 8 |
| | | 60 | 2 | | | 8 |
| | Perfusion with saline | 15 | < 2 | | | < 2 |
| | | 30 | < 2 | | | < 2 |
| | Elution in incubator | 30 | 12 | | | 32 |
| | | 60 | 8 | | | 32 |

* = 0 minute means the first 4 ml sampling.
** = The values are the reciprocals of HA titres.
— = Not tested.

The evidence obtained in the experiments has clearly shown that virus was adsorbed onto, and eluted from, the vascular endothelium. The HA test demonstrates high concentrations of virus only and in order to elucidate the finer details of this interaction between virus and vascular endothelium, the more sensitive infectivity titration method must be resorted to. Since this latter method is capable of demonstrating quite small amounts of virus, the procedure had to be slightly modified. Two representative experiments of this type are described in the following.

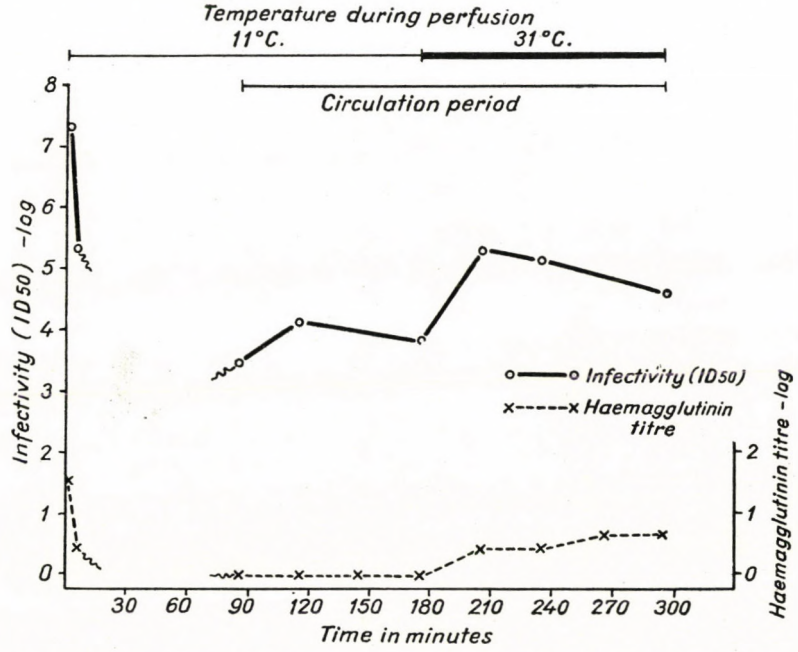


*Fig. 1.* The adsorption and the elution of PR8 virus in the hepatic vessels of rat

A rat liver, prepared as described above, was perfused with physiological saline at room temperature for 60 minutes and then for 10 minutes with saline containing 0,62 per cent formaldehyde. Then chilled physiological saline was circulated in the hepatic vessels for 60 minutes with the double purpose of removing the formaldehyde and to chill the liver. Subsequently, as described, 40 ml of the PR8 allantoic fluid virus diluted at a ratio of 1 to 5 was perfused under pressure. The last 2 to 3 ml of the virus material dripping out after passing through the liver were pooled separately to be used later for titration of virus. After this, 300 ml of chilled saline were passed through the liver preparation and the washing fluid was discarded. This procedure took 80 minutes. After the hepatic vessels were thoroughly washed, 40 ml of chilled saline were circulated in the system by the described method. The temperature

of the outflowing saline was 11°C. From the beginning of the actual experiment (i. e. when perfusion with the virus-bearing fluid had been started) until the 175th minute the circulating fluid was chilled. In this period, between the 85th and 175th minutes, three samples were taken for titration of virus. Then the glass coil was taken out of the melting ice and placed into a 37°C water bath. The circulating fluid was kept warm until the 295th minute, when the experiment ended. In this period when the temperature of the fluid dripping out of the liver was 31°C, a number of samples were again taken from the perfusion fluid.

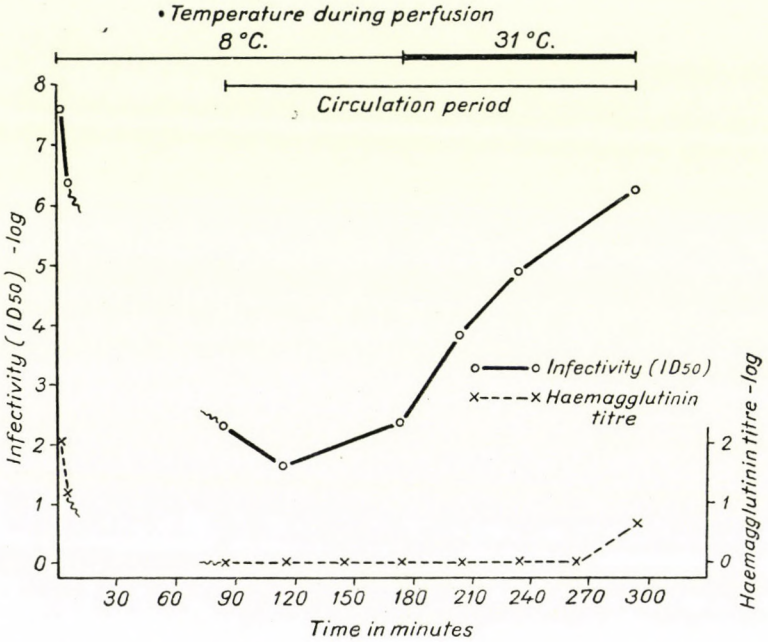


*Fig. 2.* The adsorption and elution of PR8 virus in the hepatic vessel of guinea-pig

The results of the experiment carried out with the rat liver preparation are shown in Fig. 1.

It is seen that, both the infective and HA titres of the virus material that had passed through the liver were significantly reduced.

The sample taken 90 minutes after washing, when the circulation of the allantoic fluid virus had just begun, contained virus in a titre of only $10^{-3,5}$ as compared to the initial titre of $10^{-7,33}$. During perfusion with cold fluid, the titre rose slightly, then became insignificantly reduced again. The difference between the two values was just within the limits of error of the procedure. On warming up the circulating fluid, considerable amounts of virus were liberated, as shown by the rise in both the infectivity and the HA titres. The subse-

quent slow reduction in infectivity titre was most probably due to a thermal inactivation of the virus.

The experiment was reproduced under identical conditions with guinea pig liver (Fig. 2), using the same PR8 virus (40 ml of allantoic fluid, diluted at a ratio of 1 : 5). It can clearly be seen in Fig. 2 how the virus was adsorbed even during perfusion with cold fluid and how it was gradually released, particularly on warming up. At the end of the experiment even the HA method revealed the presence of virus.

## *Discussion*

Liver preparations, from which the blood had been removed, were perfused with dilute formaldehyde through a cannula inserted into the portal vein and then thoroughly washed. After such treatment the livers bound influenza and ND viruses gradually. In support of the firm attachment of virus is the fact that elution with cold saline failed to remove it from the liver. The disappearance of virus on perfusion with cold fluid is due to the adsorptive action of the hepatic vascular endothelium. The mechanism involved is similar to that observable with adsorption onto erythrocyte or onto pulmonary epithelium, where the virus can also be released from the surface by increasing the temperature prevailing in the system.

Thus, according to the above observations, the internal surface of blood vessels is capable of adsorbing virus in a characteristic manner. The elution resulting from an increase in temperature is thought to be due to the activity of an assumed receptor-destroying enzyme of the virus. On the basis of the experiments it could, however, not be decided whether the virus-receptor substance is localized on the surface or in the connective substance of the vascular endothelium. The existence of such an inter-endothelial substance has been generally accepted, the more so, as it can be demonstrated by adequate histological methods (silver impregnation).

Whether the endothelial cells themselves, or the intercellular connective substance, are the subjects of the receptor-destroying enzyme presumably possessed by the virus, it may rightfully be asked whether this would be of consequence on the permeability of vessels and, *ceteris paribus*, is this action not one of the factors responsible for the well-known toxic character of influenza virus. The rat, rabbit and guinea pig, which are otherwise not susceptible to influenza virus, develop extensive lesions, or even die, on inoculation with large amounts of virus. There are sometimes quite striking circulatory manifestations. For instance, influenza virus injected into the anterior chamber of the rabbit's eye will cause corneal opacity and moderate iritis. The influenza virus inoculated into the rat brings about a considerable fall of blood pressure

(*Chang* and *Kempf*, 12). Even in human pathology the cardio-vascular effects of influenza virus are known phenomena [13, 14].

With respect to this problem, the observations made by *Henle* and *Henle* [15] are particularly significant. They inoculated large amounts of influenza virus, intravenously or intraperitoneally, into mice and at autopsy found in the liver focal necrosis, and in the spleen hyperaemia, degeneration of Malpighian bodies and in some cases petechiae. The latter were not infrequent in the stomach either. In addition, considerable pleural effusion could also be detected.

It seems justified to assume that one of the factors involved in the development of the above s mptoms is an alteration of vascular permeability, arisen perhaps in consequence of an enzymic action of the influenza virus. The assumption is rather tempting and would be even more so, had it not been reported by *French* and *Ada* [16] that very large doses (50 000 U per guinea pig) of highly purified receptor destroying enzyme isolated from cholera vibrio cultures were not toxic. The complicated structure of the influenza virus may by no means be considered identical with the enzyme of the cholera vibrio. Consequently, the above observation by itself does not suffice to discredit the assumption that the influenza virus has a direct injurious effect on vascular endothelium.

## SUMMARY

The portal vein was cannulated in the guinea pig, rat or mouse and the liver perfused with saline and thus rendered free of blood. The organ preparation so obtained was perfused with dilute formaldehyde, which was then carefully washed off. Subsequently, allantoic fluid containing PR8, Lee, Swine, or ND virus was circulated in the vessels of the liver preparation. During this perfusion with cold fluid large quantities of virus were gradually adsorbed. On increasing the temperature of the system by heating to above 30°C or by circulating body-warm physiological saline in the hepatic vessels, part of the bound virus became gradually released. Thus, in the animal species mentioned the viruses examined were adsorbed onto, and eluted from, the internal surface of the vascular endothelium in a manner characteristic for the influenza and related viruses. On this basis it may be assumed that the surface of the vascular endothelium coating the lumen of vessels contains a virus-binding receptor substance. It is suggested that a direct action on vascular endothelium may be one of the factors responsible for the toxicity of influenza virus.

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## СПОСОБНОСТЬ ЭНДОТЕЛИЯ МЛЕКОПИТАЮЩИХ ЖИВОТНЫХ К СВЯЗЫВАНИЮ ВИРУСА ГРИППА

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Р е з ю м е

Авторы производили обескровливание печени морских свинок, крыс или мышей путем перфузии соленой водой через стеклянную канюль, введенную в воротную вену. Через кровеносные сосуды полученного таким образом препарата пропускали жидкий раствор формалина, затем тщательно вымывали их от формальдегида. После этого в кровеносных сосудах препарата печени вводили в циркуляцию аллантоисную жидкость, содержащую вирусу PR8, Lee, Swine или ND.

В процессе этой холодной перфузии наличие вируса в жидкости постепенно и в большом количестве сокращалось. Если препарат печени подогревали более, чем до 30°, или через кровеносные сосуды пропускали соленую воду температуры тела, часть связанного вируса постепенно освобождалась. Таким образом, на эндотелии кровеносных сосудов упомянутых животных исследуемые вирусы адсорбируются и элюируются характерным для гриппа и родственных ему вирусов образом. На основе вышеуказанного можно предполагать, что поверхность эндотелия, выстилающего просвет кровеносных сосудов, содержит связывающие вирус рецепторы. В результате наблюдений авторы считают возможным, что один из факторов токсичности вируса гриппа должен разыскиваться может быть именно в оказываемом им непосредственно на кровеносные сосуды действии.