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The Electrophoresis of the Neurotropic Virus of Horsesickness and its Neutralizing Antibodies in Low Concentration.

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THE lack of reports on quantitative investigations into the mobility of virus particles and their antibodies in an electrical field must be ascribed to failure to develop a technique suitable for adaptation to that type of work. Recently the moving boundary method used by Tiselius (1930, 1937) in his studies on the electrophoretic properties of proteins has been used to supplement the previous purely quantitative observations that had been made. For instance Bourdillon and Lennette (1940) have reported their results with the complement fixing antigen of human influenza using the Tiselius apparatus. Prior to this, the author (Polson 1940) published a short note on the electrophoresis of the virus of horsesickness and briefly described the apparatus used. Essentially this is a simplified modification of the Tiselius apparatus and has been found eminently suited to the purpose for which it was designed. The following report records observations made on the electrophoresis of virus, its neutralizing antibody and neutral serum virus mixtures in low concentrations.

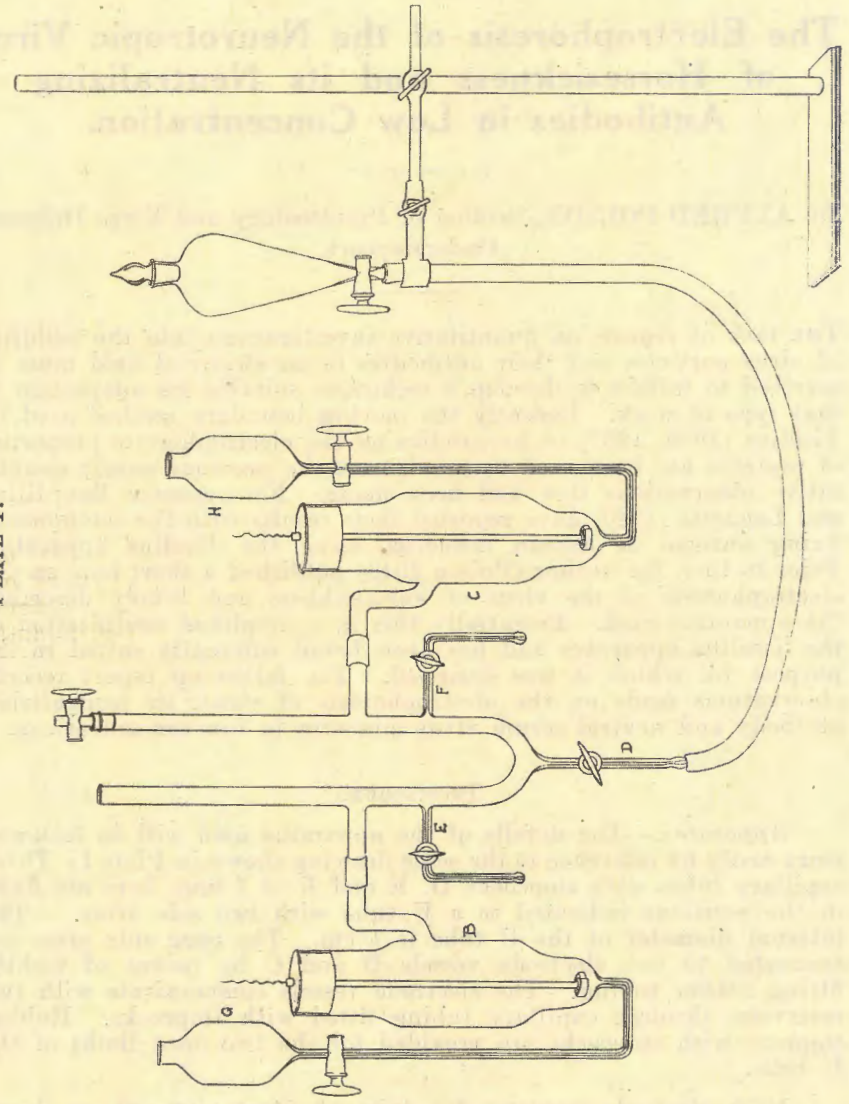
TECHNIQUE.

Apparatus.—The details of the apparatus used will be followed more easily by reference to the scale drawing shown in Plate I. Three capillary tubes with stopcocks D, E and F of 1 mm. bore are fixed in the positions indicated to a U tube with two side arms. The internal diameter of the U tube is 1 cm. The open side arms are connected to two electrode vessels B and C by means of tightly fitting rubber tubing. The electrode vessels communicate with two reservoirs through capillary tubing fitted with stopcocks. Rubber stoppers with stopcocks are provided for the two open limbs of the U tube.

Each electrode consists of a strip of silver plate 40 cm. long, 1 cm. wide and 0·5 mm. thick, rolled into a coil 4 cm. in diameter. Fused to the central end of the coil is a length of silver wire which in turn is fused into glass capillary tubing. The capillary tubing which is essential to form a rigid electrode easily maintained in position without vibration passes through a rubber stopper selected

to fit the mouth of the electrode vessel. Before use the electrodes are coated with silver chloride. This is done by attaching them to the anode of an electrical circuit and passing a current of 5 milli amps for 72 hours while immersed in a saturated solution of potassium chloride.

PLATE I.



Prior to erection, the apparatus is cleansed and all traces of virus removed by washing with alcohol followed by sterile distilled water. By means of an arrangement of retort stands and clamps the U tube with its attachments is firmly fixed in a vertical position, care being taken that the capillary tubes E and F are on the same horizontal

level. The U tube, electrode vessels, and the attached capillaries up to the stop cocks, are filled with the buffer solution to be used in this particular experiment. The reservoirs G and H are filled with a $^1/_{10}$ saturated potassium chloride solution. The electrodes are carefully lowered into the buffer to the bottom of the vessels, the rubber stoppers tightly fitted and, if necessary, securely tied in position. The potassium chloride solution is allowed to run into the electrode vessels from the reservoirs until the silver electrodes are just completely covered, the excess of buffer being allowed to escape through stopcock D; the line of demarcation between the potassium chloride and buffer is easily seen. The apparatus together with the fluid to be subjected to electrophoresis is allowed to stand until temperature equilibrium is attained. By means of a manometer arrangement the fluid is allowed to run in very slowly through D to form a sharp boundary between fluid and buffer at the level of E. and F. Prior care should be taken to pipette sufficient buffer from the upper ends of the U tube to allow for the necessary displacement. If by any chance a sharp boundary is not formed between the two liquids this may be rectified very simply by allowing fluid to run out drop by drop from E and F until the desired degree of sharpness is obtained. Finally, to ensure that none of the fluid under investigation has been in contact with glass above the line of separation from the buffer the levels are raised about 1 cm. above E and F by allowing more liquid to run in slowly through D. The open ends of the U tubes are now closed by shutting the stopcocks.

Precautions.—Vibration and the convection currents due to unequalities in temperature are sources of serious error in work of this nature. Consequently the apparatus must be mounted on a firm steady table preferably inside a celluloid box which will eliminate the effects of air currents. It is highly desirable that the temperature of the room should be thermostatically controlled; the major portion of the work to be described has been carried out in a room accurately controlled at a temperature of 7° C.

Care must be taken that when the apparatus is filled temperature equilibrium has been attained. For this reason preliminary dialysis should be carried out, and all fluids to be used should be stored in the room in which the apparatus is set up and the experiment is to be run.

The electrodes must be immersed in tenth saturated potassium chloride. If a saturated solution is used the rapid diffusion of the salt into the buffer causes a decrease in volume which may upset the entire experiment.

The maintenance of a sharp boundary between the fluid to be electrophorized and the buffer in the U tube is made possible by ensuring that the specific gravity of the fluid is higher than that of the buffer.

Buffers.—The buffers used in each experiment are indicated in the text. The pH determinations were made by a hydrogen electrode and a slide wire-potentiometer.

Electrical Current.—To avoid heating and the consequent production of convection currents very small electrical currents, usually not greater than 1 milli-amp, were used. This was supplied by an Exide dry-cell battery. However, for experiments of short duration, currents up to 3 milliamperes have been used with entire satisfaction more particularly when carried out at a low temperature.

Virus.—Different strains of neurotropic horsesickness virus were used as indicated in the respective experiments. Virus was obtained from the brains of mice destroyed *in extremis* after intracerebral infection. Usually four brains were emulsified in 40 c.c. of 10 per cent. normal horse serum saline. In experiments where it was necessary to avoid the use of serum 1·5 per cent. cane sugar was added to a saline emulsion to ensure the production of a high specific gravity. Preliminary clarification was carried out by centrifugation at 3,000 revolutions per minute for 20 minutes. The turbid supernatant fluid was dialysed for 48 hours in cellophane bags against the buffer to be used. After dialysis the emulsions were spun for 20 minutes at 9,000 revolutions per minute and then passed through a gradocol membrane with an average pore diameter of 800 mμ to ensure bacterial sterility. Care was taken during all manipulations after dialysis that a minimum of evaporation took place so that the percentage salt concentration would not be altered.

Electrophoresis.—Two methods of determining not only the direction but the rate of migration of the virus or antibody were used.

1. After passage of the current for a specified time samples were collected at various levels above the initial emulsion-buffer boundary.

2. At regular intervals after the commencement of the experiment samples were collected at a point say 2 cm. above the boundary.

The presence of virus was determined by the intracerebral injection of mice. In doubtful cases the subsequent diagnosis of horsesickness was confirmed by subinoculation and adequate bacterial sterility tests. Antibody was detected by the results of the injection of mice after the sample had been mixed with an emulsion of known virus titre and incubated for 2 hours at 37° C. Usually the neutralization test was adjusted so that the sample was required to neutralize not more than 10 M.L.D's. of virus.

Collection of Samples.—The most satisfactory method of collecting samples is to use a long, finely drawn glass capillary pipette having the end cut sharp and square. Remove the rubber stopper with its stopcock from the limb of the U tube, close the end of the pipette, and lower the capillary into the fluid up to the required level. Open the end of the pipette, fluid will enter very slowly under the hydrostatic pressure without causing any disturbing current and when an adequate volume has been collected carefully withdraw the pipette. By using any simple sliding arrangement and by selecting capillary pipettes that have been drawn perfectly straight it is easy to lower the pipette into and withdraw it from the fluid without causing any disturbance. Care should be taken that

the level of the fluid in the closed limb of the U tube is close to the bottom of the rubber stopper so that there will be little or no displacement or movement due to compression of the air. The volume of the samples collected was usually 0·2 c.c.

The viscosities of the buffers were determined by means of the Ostwald capillary method at the same temperatures at which the electrophoresis experiments were made.

A. Electrophoresis of Different Strains of Neurotropic Horsesickness Virus.

To avoid repetition of similar tables in the text full details of only two typical experiments are given.

Experiment I.

An emulsion of virus strain 1180 in 10 per cent. serum saline was prepared according to the technique described above. The conditions under which electrophoresis was carried out were the following:—

A = current strength 1·63 milliamps.

T. = temperature of electrophoresis 6·2° C.

V = applied tension 80 volts.

X = conductivity $1 \cdot 62 \times 10^{-3}$ ohm^{-1}.

F = potential gradient 1·3 volt/$cm.^{-1}$.

q = cross section of the U tube 0·78 $cm.^2$.

t = time of electrophoresis 5 hours.

Buffer 0·00066 M KH_2PO_4; 0·0126 M Na_2HPO_4; pH 8·0.

Table 1.

Presence of Virus at Levels after Electrophoresis.

Distance from Original Boundary.	Result in Mice.
4·0 cm.	0; 0; 0;*
3·75 cm.	0; 0; 0;
3·50 cm.	6; 7; 0;
3·25 cm.	6; 6; 7;
3·00 cm.	5; 6; 6;
2·75 cm.	6; 6; 7;
2·50 cm.	5; 6; 7;
2·25 cm.	5; 5; 7;

* The numbers indicate the days after injection on which the mice died. 0 = survival. × = Death from some cause other than horsesickness, e.g. injury.

Result.

The direction of the migration of the virus was anodic.

From Table 1 it is evident that the level of the virus under electrophoretic influence had moved from the original buffer-emulsion boundary to a point between 3·50 and 3·75 cm. above that boundary, i.e., the highest active and the lowest inactive level. It may be assumed then that the actual level is the mean between these points i.e., that the virus had migrated a distance of 3·625 cm.

The mobility μ may be calculated from the formula $\mu = X/Ft$. In this equation in turn the value of the potential gradient F is calculated from the formula deduced by Tiselius (1930) viz., $F = A/qx$.

Substitution in these two formulae shows that under the conditions of the experiment the mobility of the virus was $15{\cdot}5 \times 10^{-5}$ cm.^{2} sec.$^{-1}$ volt^{-1}.

For comparative purposes this calculated mobility may be adjusted to that in distilled water at 20° C. by using the formula $\mu_{20} = \mu_T \frac{\eta_T}{\eta_{20}\,°C.}$ where η_T, μ_T and η_{20} μ_{20} are the viscosity and mobility at T° C. and 20° C. respectively. The adjusted mobility, therefore, becomes $22{\cdot}7 \times 10^{-5}$ cm.^{2} sec.$^{-1}$ volt^{-1}.

Experiment II.

In this experiment a second virus 449 was electrophorized at pH 7·83. The second method of sampling was used, viz., taking samples at a level 3 cm. above the original buffer emulsion boundary at intervals of 30 minutes. The conditions under which the experiment was run were the following:—

A = 2·37 milliamps.
T = 24·6° C.
V = 80 volts.
$X = 2{\cdot}90 \times 10^{-3}$ ohms^{-1}.
F = 1·05 volts cm.$^{-1}$.
q = 0·78 cm.^{2}.
Buffer 0·00007 M KH_2PO_4; 0·0126 M Na_2HPO_4; pH 7·83.
distance of migration of virus = 3 cm.

TABLE 2.

Time in Hours.	Effect on Mice.
2·0	000
2·5	000
3·0	560
3·5	566
4·0	556
4·5	455
5·0	555

TABLE 3.

Electrophoretic Mobility of 3 Strains of Virus.

Migration Anodic.

	Strain 1180.			Strain OD.			Strain 449.		
	I.	II.	III.	I.	II.	III.	I.	II.	III.
A = Current in milliamps	1·43	1·26	4·875	0·82	1·50	1·63	1·625	2·0	2·37
T = Temperature °C.	6·5°	10°	8°	7°	6·8°	6·3°	16·5°	17·0°	24·6°
X = Conductivity = 10^{-3}ohm^{-1}	1·13	1·482	1·80	1·21	1·40	1·62	1·53	2·61	2·90
F = Potential gradient volt cm.$^{-1}$	1·63	1·096	0·555	0·868	1·38	1·30	1·36	0·98	1·05
t = Time in hours: Inactive	—	—	—	—	—	—	2·5	3·0	2·5
t = Time in hours: Active	—	—	—	—	—	—	3·0	3·25	3·0
t = Time in hours: Mean	6·75	3·0	5·0	3·0	4·66	5·0	2·75	3·125	2·75
Distance cm.: Inactive	3·0	1·75	1·0	1·0	2·5	3·75	—	—	—
Distance cm.: Active	2·5	1·5	0·75	0·75	2·25	3·50	—	—	—
Distance cm.: Mean	2·75	1·625	0·875	0·885	2·375	3·625	2·0	2·0	3·0
Buffer: KH_2PO_4 M	0·0117	0·0045	0·0006	0·01073	0·0052	0·0006	0·00801	0·00354	0·0007
Buffer: Na_2PHO_4 M	0·0017	0·00883	0·0127	0·0026	0·00813	0·0127	0·00532	0·00979	0·0126
Buffer: pH	6·20	7·11	8·02	6·35	7·02	8·01	6·64	7·24	7·83
μ = Mobility at T °C. cm^2. sec^{-1}volt^{-1}	$7·0 \times 10^{-5}$	$13·7 \times 10^{-5}$	$21·2 \times 10^{-5}$	$9·3 \times 10^{-5}$	$9·3 \times 10^{-5}$	$15·6 \times 10^{-5}$	$14·9 \times 10^{-5}$	$17·4 \times 10^{-5}$	$28·8 \times 10^{-5}$
μ 20° C. = Mobility at 20 °C. cm^2 sec^{-1}volt^{-1}	$10·16 = 10^{-5}$	$17·6 \times 10^{-5}$	$29·7 \times 10^{-5}$	$13·3 \times 10^{-5}$	$16·2 \times 10^{-5}$	$22·7 \times 10^{-5}$	$15·9 \times 10^{-5}$	$18·27 \times 10^{-5}$	$25·9 \times 10^{-5}$

Result.

Direction of migration was anodic.

The time required to move a distance of 3 cm. is taken as the mean between the time when the last inactive and the first active sample was collected, i.e., the mean between 2·5 and 3·0 hours = 2·75 hours.

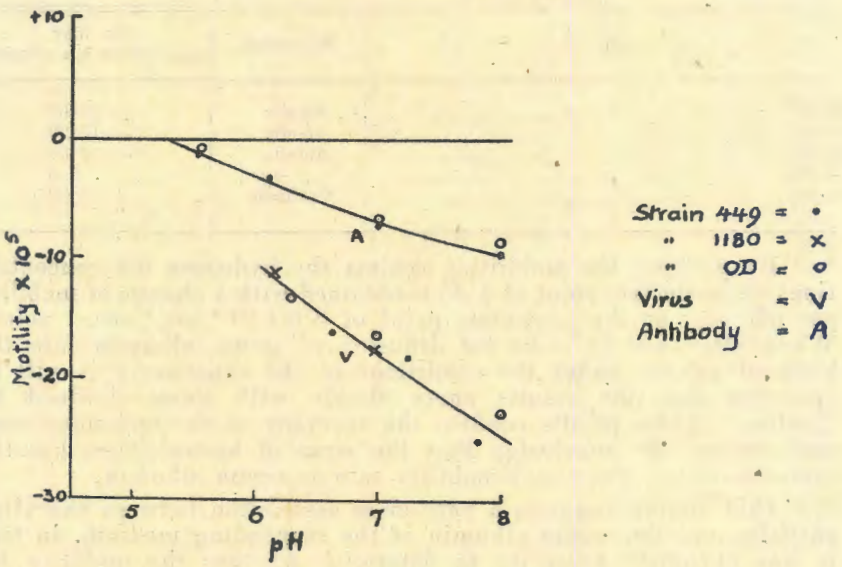
By substitution in the formulae cited above $\mu_{24\cdot6} = 28\cdot8 + 10^{-5}$ cm.^{2} sec.$^{-1}$ volt^{-1}. Corrected to the calculated mobility in distilled water at 20° C. the value becomes $25\cdot9 \times 10^{-5}$ cm.^{2} sec.$^{-1}$ volt^{-1}.

Having considered in detail the results obtained with typical examples of both methods of experimentation the data collected from electrophoresis of three antigenically different strains of virus may be tabulated. These are shown in Table 3.

In Plate II Curve V the various mobilities as determined in the above series of experiments are plotted against the hydrogen ion concentrations.

PLATE II.

Comparison of the Mobilities of Virus and Antibody.



Result.

Consideration of the curves shows that there is no significant difference in mobility of the different strains of virus used over the range of pH in the experiments. Since the virus is unstable at pH's below 5·99 the isoelectric point can be determined only by extrapotation of the curve. This gives an isoelectric point of 4·8 and a change of mobility with pH at that point of approximately $9\cdot0 \times 10^{-5}$ cm.^{2} sec.$^{-1}$ volt^{-1}.

It is interesting to note that Tiselius (1930) gave a figure of 4·88 for the isoelectric point of serum albumin and a change of mobility with pH at the isoelectric point of $9\cdot1 \times 10^{-5}$ cm.^{2} sec.$^{-1}$ volt^{-1}.

Tiselius' figures agree so closely with those determined for horse-sickness virus as to suggest that the differences are due to experimental errors which will always occur when a biological test is necessary to determine the presence or absence of virus in any given sample. In order to test the reliability and accuracy of the technique used similar determinations were carried out on serum albumin.

Serum albumin prepared and crystallized according to the method of Kekwick (1938) was dialysed against buffers of different hydrogen ion concentrations the most suitable buffer being found to be Na_2HPO_4—citric acid. Under the conditions of electrophoresis the migration the serum albumin was determined by judging the change in position of the maximum gradient in refractive index between the solvent and solution. It must be stated that concentrations of serum albumin as high as 2 per cent. were used in order to obtain a sharply defined difference in refractive index. Current densities of the order of 1 milliamp per cm.^{2} were used to eliminate errors due to convection currents. The calculated mobilities determined for serum albumin are shown in Table 4.

TABLE 4.

pH.	Migration.	Mobility $\mu_{20} \times 10^5$ cm.^{2}sec.$^{-1}$volt^{-1}
6·10	Anodic	− 10·60
5·85	Anodic	− 10·20
5·59	Anodic	− 7·56
4·76	—	0·0
4·40	Cathodic	+ 2·80

By plotting the mobilities against the hydrogen ion concentrations an isoelectric point of 4·80 is obtained with a change of mobility per pH unit at the isoelectric point of $8{\cdot}6 \times 10^{-5}$ cm.^{2} sec.$^{-1}$ volt^{-1}. When allowance is made for diffusion of serum albumin into the buffered solvent under the conditions of the experiment it will be conceded that the results agree closely with those obtained by Tiselius. These results confirm the accuracy of the technique used and support the conclusion that the virus of horsesickness has the same isoelectric point and mobility rate as serum albumin.

This finding suggests a very close association between the virus particles and the serum albumin of the suspending medium, in fact it was obviously necessary to determine whether the mobility for which the virus particles was credited was not actually the mobility of serum albumin molecules which dominated an albumin-virus complex. In this connection it is interesting to note that Kligler, Olitsky and Aschner (1931) in their work on the electrophoresis of fowl-pox virus found that, in the presence of proteins, the virus carried a negative charge over the range pH 5·2 to 9·8. On the other hand in the absence of proteins, which were eliminated by adsorption on and elution from kaolin, the virus wandered to both the positive and the negative pole at any given pH; this phenomenon was most marked in neutral solutions. The difference in mobility of the virus in the protein-containing and protein-free solutions indicated that the electrical charge measured was that of a protein

adsorbed onto the virus. Unfortunately the technique of Kligler and his associates is open to criticism. For instance neither the current densities nor the resulting rise in temperature were controlled yet it is known that a current not greater than 4 milliamps per square cm. is permissible at room temperature if convection currents due to overheating are to be avoided; only at lower temperatures (3°—5° C.) are higher current densities permissible (Tiselius 1938). Further, the use of agar bridges in the apparatus introduces the possibility of a source of serious error due to endosmotic flow of the solution under examination. Finally it is not possible to check the apparatus and technique employed since no other substance is known which will migrate to both the negative and positive poles at a given pH under the influence of electrophoresis.

Up to the present time it has not been possible to devise a critical experiment which will finally clear up the electrophoretic activity of a virus particle. However, several experiments were conducted with the object of throwing some light upon the problem.

I. Pure haemoglobin was prepared from horse blood according to the method of Ettisch and Grosscurth (1933). The brains of 4 mice destroyed *in extremis* as a result of infection with horsesickness virus strain 449 were emulsified into 50 c.c. of a 1 per cent. solution of this haemoglobin in physiological saline. The emulsion was dialysed overnight against a buffer consisting of 0·0065 molar KH_2PO_4 and 0·0065 molar Na_2HPO_4, pH 6·8. After dialysis the emulsion was centrifuged at 10,000 revolutions per minute for 20 minutes and then filtered through a gradocol membrane having an average pore diameter of 800 m μ. The filtrate was subjected to electrophoresis and an anodic migration with a mobility of 15×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1} at 20° C. was determined. This figure agrees closely with that determined for the mobility in serum saline at pH 6·8. The haemoglobin being at its isoelectric point did not migrate and no virus could be detected in the cathodic limb of the U tube at the original virus boundary.

II. In the medium of serum globulin at pH 7·02 the virus had the same mobility as in serum saline and no virus could be detected in association with the globulin in the cathodic portion of the U tube.

III. Attempts have been made to free infective brain emulsions of protein by tryptic digestion. This work is still in progress and will form the subject of a further report at a later date. At this stage it may be stated that after digestion the rate of mobility did not differ from that determined in serum saline.

All these experiments serve merely to demonstrate the close association of the virus with the serum albumin. Unfortunately they do not clear up the point as to whether the electrophoretic activity of the virus is a property *per se* or is due to an intimate linkage with the albumin.

B. Electrophoresis of the Neutralizing Antibody of Horsesickness Virus contained in the Serum of Immunized Horses.

Technique.—The apparatus and technique described for the electrophoresis of virus was used in this series of experiments.

Serum.—Antiserum was obtained from two sources:—

1. From a horse, 21239, which had been immunized against strain OD only.
2. From a horse, 22000, which had been immunized with routine polyvalent vaccine containing strains 449, 1,180, 0, Vryheid, OD and KA.

Prior to electrophoresis the sera was dialysed in the cold overnight against the buffer to be used, and were clarified by centrifugation at 3,000 revolutions per minute for 20 minutes.

Detection of Antibody.

Samples in 0·2 c.c. amounts were collected at different levels after electrophoresis for a specified time or at different intervals of time at a specified level above the original serum buffer boundary. The serum samples were added to 0·8 c.c. of a clarified emulsion of infective mouse brains in serum saline, the concentration being adjusted by previous titration so that 0·05 c.c. contained 50 minimal infective doses of virus. The virus-antibody mixtures were incubated for 2 hours at 37° C. and then injected intracerebrally into mice. The boundary of migration was taken as the mean between the last sample which failed to neutralize the virus and the first sample which afforded protection, the index of protection being the survival of half of the mice injected. An example of a typical experiment is given in Table 5, the conditions under which electrophoresis was carried out being the following:—

Current 0·95 milli amps.
Temperature 7·0° C.
Applied tension 80 volts.
Conductivity $1{\cdot}57 \times 10^{-3}$ ohm^{-1}.
Cross section of U tube 0·78 $cm.^2$.
Time 8·33 hours.
Buffer 0·00066 KH_2PO_4, 0·0126 M Na_2HPO_4, pH 8·01 M.

TABLE 5.

Presence of Antibody at Various Levels after Electrophoresis. Migration Anodic.

Distance from Original Boundary.	Result in mice.
2·0 cm.	5; 5; 5;
1·75 cm.	5; 5; 6;
1·50 cm.	5; 6; 0;
1·25 cm.	0; 0; 0;
1·0 cm.	0; 0; 0;
0·75 cm.	0; 0; 0;
0·5 cm.	0; 0; 0;
0·25 cm.	X; 0; 0;
0·0.	0; 0; 0;

Results.

From the above it is evident that the virus had moved to a level between 1·25 and 1·50 cm. above the original boundary. Assuming the true level to be the mean between these levels, i.e., 1·325, μ is calculated as 6×10^{-5} cm.^{2}/sec./volt and μ_{20} as $8{\cdot}46 \times 10^{-5}$ cm.^{2} sec.$^{-1}$ volt^{-1}.

In Table 6 the results are given as a series of experiments on the electrophoresis of antibodies over a range of pH.

In Plate II, Curve A, the antibody mobilities are plotted against the pH.

Results.

There appears to be no difference in the rate of mobility of the neutralizing antibody of strain OD and 449. The mobility of the antibody is approximately half that of virus with an isoelectric point at approximately pH 5·3. Tiselius (1937) separated several components from serum globulin by electrophoresis and termed them Globulin α, β and γ. He determined their electrophoretic properties, and found globulin α to have an isoelectric point of pH 5·06, globulin β of pH 5·12 and globulin γ of pH 6·0. As can be seen from the above the neutralizing antibody of horsesickness virus has an isoelectric point close to that of globulin β.

C. Attempted Separation of Virus from Inactive Virus Antibody Mixtures.

Olitsky and Long (1929) reported that under the influence of electrophoresis active virus could be separated from the tissues of rabbits which had recovered from infection with vaccinia. Further, rabbits from which active virus was recovered possessed a solid immunity to reinfection, whereas rabbits from which no virus could be isolated were susceptible. Herzberg (1933) failed to confirm this work. In addition Lepine (1930) working with vaccinia, Nicolau and Kopciowska (1930) with herpes and rabies and Levaditi and Lepine (1931) with poliomyelitis, failed to demonstrate by catophoresis any persistence of active virus in the tissues of recovered animals. Kligler and Olitzky (1931) failed to separate virus from the tissues of guinea-pigs which had recovered from infection with typhus nor did they succeed in separating active virus from neutral virus anti-serum mixtures. They attributed this failure to the fact that the virus and antibody moved in the same direction in an electrical field. If Olitsky and Long's findings were correct and were applicable to all viruses it was considered that the neurotropic virus of hersesickness and the inactivating antibodies contained in serum from immune horses would provide excellent material for further detailed investigation. In addition it is known that guinea-pigs which survive an intracerebral injection of virus are resistant to subsequent infection hence there was available an abundant supply of immune tissue.

The general method of the investigation was to utilize the different rate of mobility of virus and antibody to cause a separation of virus from its antibody, the presence of virus to be demonstrated

TABLE 6.

Electrophoretic Mobility of Neutralizing Antibody.

Migration Anodic.

Conditions of Electrophoresis.	Experiment Number.						
	1	2	3	4	5	6	7
A = Current in milliamps.	0·95	0·82	0·60	0·85	1·82	1·50	0·67
T = Temperature °C.	7°	7°	6·8°	6·3°	18·5°	17°	7·2°
X = Conductivity × 10^{-3} ohm^{-1}	1·57	1·42	1·31	1·49	2·18	1·75	1·12
F = Potential gradient volt cm.$^{-1}$	0·778	0·763	0·6	0·726	1·07	1·096	0·76
t = Time in hours — Inactive	—	—	—	—	8·0	7·5	—
t = Time in hours — Active	—	—	—	—	8·5	8·5	—
t = Time in hours — Mean	8·33	8·5	6·0	5·25	8·25	8·0	4·0
Distance cm. — Inactive	1·5	1·25	0·25	1·0	—	—	0·25
Distance cm. — Active	1·25	1·0	0·0	0·75	—	—	0·0
Distance cm. — Mean	1·375	1·125	0·125	0·875	2·0	1·0	0·125
Buffer — KH_2PO_4	0·00066	0·0053	0·0129	0·00066	0·0053	0·0113	0·0129
Buffer — Na_2HPO_4	0·0126	0·0080	0·0004	0·0126	0·0080	0·002	0·0004
Buffer — pH	8·01	7·0	5·51	8·00	7·0	6·1	5·50
μ_T cm.^{2}sec.$^{-1}$volt^{-1}	6·0 × 10^{-5}	4·83 × 10^{-5}	0·96 × 10^{-5}	6·48 × 10^{-5}	6·3 × 10^{-5}	3·14 × 10^{-5}	1·14 × 10^{-5}
μ_{20}°C. cm.^{2}sec.$^{-1}$volt^{-1}	8·46 × 10^{-5}	6·76 × 10^{-5}	1·36 × 10^{-5}	9·4 × 10^{-5}	6·7 × 10^{-5}	3·39 × 10^{-5}	1·6 = 10^{-5}

NOTE.—In experiments 1, 2, 3, the monovalent antibody against strain OD was used. In experiments 4, 5, 6, and 7 polyvalent antiserum was electrophorized and strain 449 was used as antigen in the serum virus neutralization tests.

by the intracerebral injection of mice. Sufficient immune serum, of a titre which had been determined accurately by previous serum virus neutralization tests, was added to an infective brain emulsion so that all virus would be just inactivated. The neutral mixture was dialysed against the buffer at pH 8·02 and then subjected to electrophoresis for 8 hours. It was calculated that under the conditions of the experiment the virus would have moved to a level 1·75 cm. above the original boundary whereas the antibody would have migrated only 0·8 cm. No active virus could be detected at any level. This negative result was confirmed repeatedly over a range of pH's. Similarly, a further series of experiments were carried out in which samples were collected at a level 2 cm. above the original boundary at regular intervals of time. Again no active virus could be recovered though the different rates of migration of the virus and antibody could be calculated accurately.

Similar experiments with emulsions of brain and spleen from immune guinea-pigs also yielded negative results.

Finally the technique used by Olitzky and Long (1929) was followed. Essentially this consists of collecting virus and antibody in agar bridges connected to an electrical circuit. Again the results were completely negative.

From this series of experiments it was concluded that by the methods employed it has not been possible to separate active virus from neutral serum virus mixtures or to recover virus from the tissues of immune guinea-pigs that had recovered from previous infection.

Summary.

1. By means of a modified form of the moving boundary method of electrophoresis of Tiselius, it was found possible to measure the rate of migration as well as the isoelectric points of viruses and their neutralizing antibodies in low concentrations.

2. The measurements were carried out on three antigenically different strains of horsesickness virus. It was found, within experimental error, that all three strains have the same isoelectric point as well as the same change of mobility with pH.

3. The similarity between the isoelectric points and the mobilities of serum albumin and horsesickness virus suggests a close relationship between the albumin and virus. The serum albumin can either be adsorbed on to the virus or the virus can have the same chemical composition as the albumin.

4. The mobilities and isoelectric points were determined of the neutralizing antibidies of strains 449 and OD. The same isoelectric point and the same change of mobility with pH were found for these two substances.

5. Attempts were made by means of electrophoresis on the separation of active virus from neutral virus antibody mixtures but with no success.

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