STUDIES IN ULTRAFILTRATION.

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(Received for publication, May 6, 1927.)

The limitations of the term "filtrable virus" have been well defined in a recent critical review on these agents by T. M. Rivers (1), who points out the chaos that has reigned in the loose classification of a variety of pathogenic agents on the uncertain basis of filtrability. His review renders it unnecessary for us to reiterate the difficulties that are involved in any work that deals with the nature of agents that—as he asserts—have never been cultivated in the absence of living cells and can be recognized only by their biological or pathogenic effects. Nevertheless, it would be a considerable step toward clearer understanding could we obtain information concerning the actual sizes of some of those active and pathogenic agents, which pass through filters that hold back the smallest bacteria and which are either too small to be seen with the microscope or, for reasons of chemical constitution, have defied methods of staining.

We shall not refer to the complications that render the process of filtration an uncertain method. Stuart Mudd (2) and others have thoroughly discussed these matters in recent publications, and since our work deals entirely with filtration through collodion membranes, considerations which apply to the ordinary bacterial filters of the Berkefeld, Mandler, Chamberland types are omitted.

Our present communication deals with an endeavour to approach the problem of the magnitude of some of the so called "filtrable viruses" by measuring them against the permeability of graded filters made of collodion by a method more or less analogous to that by which Bechhold (3) attempted to establish a scale of sizes for various substances ranging from crystalloids to Prussian blue. Bechhold used formalin-fixed gelatin and acetic acid collodion filters under considerable pressures. As will be seen, our own methods have aimed particularly at avoiding the high pressures employed by him.

The difficulties of the problem are such that it is of course impossible to arrive at any definitions of actual size, but it does seem to us possible by the methods used to formulate a conception of relative size which may have no inconsiderable biological significance, for the relative size of some of these active agents might well lead to a clarification of disputed points.

Between the smallest colloids and the smallest visible formed living particles there is an enormous range, somewhere within which there may be a transitional stage manifest as the "ferments" and enzymes, substances that partake in some of their activities of the properties of living matter. The difficulty of differentiating between invisible living particles and enzyme-like substances has become one of the recent quandaries of scientific speculation in connection with the so called "bacteriophage" of Twort and d'Hérelle, a difficulty for the solution of which crucial experimental methods are still lacking. It has likewise suggested itself to almost every intelligent worker with viruses such as those of herpes, chicken sarcoma, etc., that it might well be that these agents are not living cells at all, but that all the phenomena in which they are involved could be explained by the assumption of a specific "cytophage" acting upon tissue cells as do the lytic principles of d'Hérelle upon bacteria. Could we with reasonable accuracy compare the relative magnitudes of some of these viruses with known organic and inorganic colloidal suspensions, and with enzymes, the approach to understanding them should be considerably facilitated. Moreover, with knowledge increasing concerning the molecular weights of pure proteins, one might even hope for an eventual definition of the smallest size possible for a living cell capable of metabolic function.

There are, as far as we know, only a few investigations in which methods similar to our own were used for analogous purposes. One of these, by Levaditi and Nicolau (4), published in 1923, consisted in attempts to appraise the magnitudes of rabies virus, encephalitis virus (which they still considered separately from herpes virus) and a "neurovaccine" described by them. Their experiments were irregular and led to conclusions which were entirely at variance with those to which we have been led by our own experience. A later piece of work by Olitsky and Boëz (5) which appeared while we were preparing this article for press, concerns itself, among other things, with the filtration of the virus of foot-and-mouth disease. While their methods were to some extent different from our own, their results indicate a

considerable degree of similarity between the virus of this disease and those which we have studied.

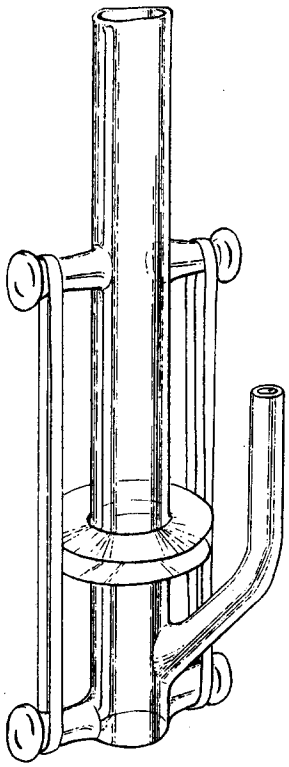
The general technique followed depended upon the production of collodion membranes in which permeability was varied according to the principles worked out by a number of investigators, those particularly consulted by us being Brown (6), Bechhold, Nelson and Morgan (7), Hitchcock (8) and Schoep (9). The principles upon which the variation of the permeability of the collodion membranes depends are as follows:

The concentration of the collodion solution; the number of coats of collodion employed; the intervals of air drying between coats; the final drying after the application of the last coat; the percentage of alcohol in which the membrane is soaked after air drying; and the length of time for which it is left in this alcohol solution. This gives a wide range of possible variation, which was at first limited in our experiments by the fact that the use of percentages of collodion less than 1 per cent resulted in membranes too fragile and soft to stand up in the filtering apparatus. This difficulty was overcome as indicated below.

While a number of different methods of carrying out this procedure were from time to time employed, the standard method finally adopted and satisfactorily utilized, throughout, consisted in making the membranes over test-tubes, carefully cleansed, soaked in distilled water and dried. These test-tubes were then slipped over a slightly tilted, motor-driven axis in an apparatus copied in a small form from one used for similar purposes on a larger scale by Dr. Edwin Cohn. The purpose of the motor used was to revolve the test-tube at a uniform rate, while the appropriate collodion solution was being slowly poured over it. Each separate covering of the test-tube in this way was designated a "coat," and a given interval was allowed before the application of a further coat by the same method. After the final coat had been applied and a stated interval again allowed to elapse, the tube was taken off the motor, immersed in diluted alcohol and left there for 30 minutes. At the end of this time it was immersed in water and left there overnight. On the following day the membrane was split with a razor by cutting along a line from one end of the tube to the other. It was then carefully peeled off and floated in water. Each of these membranes could be divided into three or more equal parts for use in the apparatus described below, and usually in our later experiments parts of the same membrane were employed for filtration at one and the same time under the same suction, and tested together for the speed with which they permitted distilled water to pass before and after the actual filtration experiment was carried out.

When it became necessary to produce membranes that would be sufficiently permeable to allow casein to pass through, and collodion

concentrations of less than 1.5 per cent were desired, we found that the resulting membranes were too soft to withstand pressures of 8 cm. of mercury or over. We finally succeeded in producing satisfactory membranes with sufficient permeability and of adequate strength by modifying the above procedure in a simple manner as follows:



TEXT-FIG. 1. The glass apparatus used for the filtrations. It was designed in our laboratory some years ago for another purpose by D. P. Morgan, Jr., and made for us by the Macalaster, Bicknell Company of Cambridge, Mass.

A sheet of Japan lens paper was wound about the cleansed and dried test-tube in a spiral manner, completely covering its surface, and was made to adhere to the test-tube by wetting it down with alcohol-ether, smoothing it and applying it without wrinkles. After this, the collodion coats were applied as usual. This technique, it is found, is useful even for membranes of lesser permeability, since thus reenforced they are more easily peeled off the tubes, and can be more safely manipulated and divided into parts without fracture.

The above technique was quite satisfactory for all filtrations in which permeabilities no greater than those necessary for the passage of collargol and casein were required. When we attempted, however, to obtain a still greater permeability the alcohol and ether method yielded membranes too soft to be manipulated with ease or subjected to any degree of pressure. We carried out our final experiments, therefore, with membranes made by the glacial acetic acid-collodion method described by Bechhold and by Schoep.

Filter paper was impregnated with acetic-collodion *in vacuo*, and the permeability varied by the percentage of gun cotton dissolved in the acetic acid. These membranes were washed for 24 hours in running water, and with them a permeability could be obtained that was sufficient for our purposes without the extreme friability of the alcohol-ether membranes.

The filtration apparatus used requires no description further than the explanatory legend under the figure included in this paper.

In actually filtering we have attempted to avoid any considerable degree of suction and so to control it that it might be both accurately measured and constant during the experiment. This was accomplished by attaching, to a water pump, a mercury trap which permitted us to vary the suction between 1 and about 20 cm. of mercury, and to hold it for any length of time at any given pressure.

Bechhold, in his work with ultrafiltration, in which he employed filter paper impregnated with gelatin and fixed in formalin or acetic-collodion, used pressures as high as 315 atmospheres and established a graded series of magnitudes for a number of colloidal suspensions. He classifies in this way twenty-three substances, the largest ranging from the crystalloids through various proteins and metallic colloids to Prussian blue. Cohn (10), after determining the minimum molecular weight of certain proteins, has estimated the relative sizes of their molecules by dialysis and ultrafiltration through membranes of graded permeability. By a combination, then, of the analytical and physical-chemical methods, he has constructed a scale in which the molecular weight of egg albumen is 33,400; serum albumen is 45,000 and casein is 192,000. Cohn summarizes the reliability of the filtration method for the determination of the dimensions of proteins by stating that: "If a membrane is made which is permeable to one protein, but not to another, then the latter may be considered the larger, provided the difference is shown not to depend upon the electrical forces involved."

It seemed to us wise to begin our work by comparing a number of the filtrable viruses, namely, that of herpes and the Rous chicken sarcoma, with a typical bacteriophage, with trypsin and with the three proteins—crystallized egg albumen, crystallized serum albumen from horse serum and solutions of purified casein. The crystallized egg albumen and serum albumen (horse) were made by the well known Sörensen method and immunologically determined in the filtrates with specific antisera. Pure casein was obtained from Dr. Edwin Cohn and was determined in the filtrates by means of a relatively weak but specific antiserum and by a ring test in which N/40 hydrochloric acid was layered over the filtrate.

I.

Preliminary Experiments.

Our first membranes were relatively impermeable. They were made by the following formula:

3 per cent collodion, 2 minute interval between coats, 6 coats, 10 minutes final drying, 30 minutes in 40 per cent alcohol, water overnight.

Membranes of this variety in a number of experiments placed in the apparatus under 8 cm. mercury suction allowed egg albumen to pass through but held back horse serum albumen and herpes virus.

A considerable number of experiments of this type were done, and it became clear to us that in developing a satisfactory technique we must take into account changes in permeability that might result from gradual increase of the suction.

An example of such an experiment follows:

Membrane C12.—3 per cent collodion, ½ minute intervals between coats, 6 coats, 10 minutes final drying, 30 minutes in 50 per cent alcohol, water overnight. Filtration at pH 7.2[1]

Suction cm. Hg—Successive filtrations, same membrane.

At 3—held back horse serum albumen.
6—let horse serum albumen through.
(*Membrane washed with water.*)
At 3—held back trypsin.
6—held back trypsin.
(*Membrane washed with water.*)
At 6—again let horse serum albumen through.

This experiment indicates that the particular membrane made as described held back horse serum albumen at 3 cm. Hg pressure, but allowed horse serum albumen to pass at a pressure of 6 cm. Trypsin was held back at the higher pressure, and the fact that horse serum albumen passed a second time at 6 cm. after the trypsin experiment had been done indicates that the trypsin was not held back because of the preliminary plugging of the membrane by the serum albumen. This would tend to indicate that trypsin in the impure condition in which we had it was larger than horse serum albumen, always assuming that at pH 7.2 it was not the electrical conditions that determined the permeability.

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[1] Hereafter, to economize space, we will describe our alcohol-ether membranes by a formula in which the order of the figures follows the sequence of the description of Membrane C 12. Thus, this membrane would be 3 per cent-½ min.–6–10 min.–30 min. 50 per cent-water overnight.

Filtration of a Mixture of Serum Albumen and Trypsin Solution.

Membrane D 5.—2.5 per cent–½ min.–6–10 min.–30 min. 50 per cent.

Mixture of Trypsin Solution and Horse Serum Albumen.

pH 7.2 at 4.5 cm. Hg—Horse serum albumen +

Trypsin −

This experiment again indicates that trypsin in the impure form in which we used it is held back by a membrane which lets through serum albumen.

Experiment Comparing Horse Serum Albumen and Casein.

Membrane E 5.—1.5 per cent–½ min.–6–2 min.–25 min. 95 per cent.

At 2 cm. Hg.

H_2O—0.25 cc. in 15 minutes.

Casein solution—0.2 cc. in 15 minutes—negative.

(Washed in water.)

Horse serum albumen—positive.

This experiment indicates that with the membrane made as above indicated casein is held back while horse serum is allowed to pass through. The measurement of the amount of water passed through at a given pressure in 15 minutes, the method adopted throughout in later experiments to determine relative permeability, shows that the casein considerably plugs the membrane; but in spite of this the horse serum albumen still came through after the membrane had been partially plugged by casein. The similarity of the isoelectric points of casein and horse serum makes it unlikely that electrical conditions have any part in the results of the experiments, and indicates that casein is larger than horse serum albumen.

Experiment Comparing Casein and Trypsin.

Membrane E 3.—1.5 per cent–½ min.–6–4 min.–30 min. 95 per cent.

At 5 cm. Hg.

H_2O—2.5 cc. in 20 minutes.

Casein—0.35 cc. in 15 minutes—negative.

(Washed with water.)

Trypsin solution—1.25 cc. in 15 minutes +++.

H_2O—1.0 cc. in 20 minutes.

This experiment shows beyond doubt that trypsin, even in the impure state in which it must have been present in our solution, was smaller than casein. For it isquite apparent that the casein plugged the membrane but that, in spite of this plugging, the trypsin that was subsequently filtered came through readily and the final water test showed that there was no gross leakage.

The foregoing experiments, then, indicate that it is possible to grade filters so that they measure particles in the order in which their molecular weights would indicate size; namely, crystallized egg albumen,

crystallized serum albumen next and pure casein the largest. While these experiments, moreover, indicate definitely that trypsin is smaller than casein, they also suggest that trypsin may be somewhat larger than serum albumen. This, however, cannot be definitely accepted because the trypsin may, in our necessarily impure solutions, be adsorbed to other constituents, possibly to a protein. Nevertheless, it is important in connection with our subsequent studies to realize that in size trypsin probably approximates to serum albumen and is definitely smaller than casein.

II.

Filtration of a Staphylococcus Bacteriophage in Comparison with Trypsin and Casein.

A staphylococcus bacteriophage, which was supplied as through the kindness of Miss Elsie Schumm, was used. To a 5 to 7 hour young culture of a susceptible staphylococcus in broth, 1/10th of the volume of a Berkefeld filtrate of the bacteriophage was added, and the mixture kept at room temperature overnight. On the next morning the culture was found to be clear and the clear fluid was again filtered through a Berkefeld candle and adjusted to pH 7.2 for the experiments. Tests for the phage were then carried out with similar young cultures on the fluid above and below the filter membrane.

Membrane K 4.—1 per cent–¼ min.–6–4 min.–30 min. 50 per cent. Divided into 3 parts simultaneously tested.

	Initial test. Water at 2 cm. Hg in 10 min.	2 cm. Hg 16 min.	Final water test for leakage. 2 cm. Hg 10 min.
	cc.		*cc.*
K 4a	1.25	Casein 0.8 cc. negative	0.15
K 4b	1.25	Trypsin 0.8 cc. positive	0.25
K 4c	1.25	*Staphylococcus aureus* bacteriophage 1.5 cc. negative	0.5

This experiment would tend to show that under the conditions of filtration bacteriophage is held up by a filter which permits trypsin to pass.

In this experiment we began a practice which is followed throughout hereafter, and which is of considerable importance. It consisted in not only measuring the flow of water per stated interval at a given pressure for each individual membrane before the filtration of a specific substance was attempted, but in adding to this a final measurement of the passage of water under the same pressure for the same

length of time, after the specific filtration had been carried out. This not only insured us against errors of gross leakages, but furnished a very definite indication of the degree to which the membrane had been plugged by the substance previously filtered. It may be noted in passing that nothing plugged the membranes quite as extensively and regularly as casein.

Membrane N 5.—1 per cent–¼ min.–6–4 min.–30 min. 95 per cent—membrane divided into 3 sections.

	H_2O, 2 cm. Hg 5 min.	2 cm. Hg 6 min.	H_2O again2 cm. Hg 5 min.
	cc.		*cc.*
N 5a	1	Casein 0.75 cc. Casein ++ by acid test	0.25
N 5b	1	Trypsin 1.0 cc. Trypsin ++	0.75
N 5c	1.5 (Bigger surface.)	Phage 1.5 cc. Phage negative	1.0

This experiment shows that under conditions which will let through both casein and trypsin bacteriophage does not pass, even though the tests with water indicated that if there were any difference between the three sections of Membrane N 5, it was in favour of greater permeability for the one through which it is attempted to pass the bacteriophage. Similar experiments confirmed this result with regularity.

Filtration of Rous Sarcoma Virus.

The Rous sarcoma virus was prepared in the following manner: Tumors freshly taken from chicks were minced and ground up thoroughly in sand, then emulsified in Ringer's solution and, in a preliminary filtration, passed through paper pulp and sand filters in the manner usually employed in working with this tumor. The resulting filtrate was the material used in our collodion membrane filtrations.

Comparison of Horse Serum Albumen and Rous Sarcoma Virus.

Membrane C 13.—3 per cent–½ min.–2–5 min.–30 min. 50 per cent.

Mixture of Rous Sarcoma Virus and Horse Serum Albumen.

Suction cm. Hg—Filtrations at pH 7.2.

At 4—held back horse serum albumen.
At 8—held back horse serum albumen.
At 12—held back horse serum albumen.
At 16—let horse serum albumen through.

Chicks were injected with portions of every filtrate except the one at 4 cm. Hg, and all were negative. Controls were positive.

This membrane did not permit horse serum albumen to pass until the pressure was increased to 16 cm. Hg. Even at this pressure, however, chicken sarcoma virus did not pass through the membrane.

Comparison of Casein and Rous Chicken Sarcoma Virus.

Membrane K 5.—1 per cent–¼ min.–6–2 min.–30 min. 50 per cent—divided into 2 parts simultaneously tested.

	Water at 2 cm. Hg 10 min.	2 cm. Hg 15 min.	Water at 2 cm. Hg 10 min.
	cc.		*cc.*
K 5a	2	Casein 1 cc. positive test	0.3
K 5b	2	Rous chicken sarcoma 1 cc. chick inoculated negative, control positive	0.5

This experiment indicates that the casein will come through a membrane which holds back the Rous chicken sarcoma. Curiously enough, however, in this case the amount of obstruction of the membrane was greater for the casein than for the sarcoma solution. It is observations of this kind that have suggested to us the necessity of controlling the electrical conditions. Namely, if the negatively charged casein is passed by Membrane K 5, this same membrane might hold up a smaller sarcoma virus if this had an isoelectric point at a pH higher than that at which we were filtering, namely, pH 7.2, and were, therefore, positively charged. This matter is controlled in other experiments below.

Membrane N 6.—1 per cent–¼ min.–6–4 min.–30 min. 90 per cent. Divided into 3 parts simultaneously tested.

	H_2O – 2 cm. Hg	Material filtered	Amount passed in 15 min. at 2 cm. Hg	Retested with water 2 cm. Hg. Amount passed in 10 min.
	cc.			*cc.*
N 6a	1	Casein solution mixed with Rous sarcoma virus	1.0 cc. Negative for casein Chick inoculated	0.5
N 6b	1	Casein solution alone	1.0 cc. Positive for casein	0.5
N 6c	1.5 (Slightly larger area.)	Rous sarcoma virus alone	1.75 cc. Chick inoculated	1.0

Before filtration these materials all tested by specific anticasein serum and those containing casein found positive. Similar controls were made with the acid test. Chick inoculations both negative. Controls positive in 10 days.

This experiment differs from the others in that we attempted to filter through one part of the membrane a mixture of casein solution and Rous sarcoma virus, filtering them separately through the two remaining sections of the same membrane. The casein tests were done both by anticasein precipitating serum and by layering N/40 hydrochloric acid over the solutions. The anti-precipitating serum showed the casein definitely in the mixture and in the casein solution, but gave no ring in the Rous sarcoma virus alone before filtration, and the acid test showed a sufficient difference between the mixture and the Rous sarcoma virus to be of comparative value.

It is interesting to note that where the casein solution was filtered alone it came through at a pressure that held it back when it was mixed with the Rous sarcoma virus, a phenomenon which in one way or another has appeared with various substances which we have tried to filter in mixtures and for which a number of explanations can be suggested but none definitely proven at the present time.

Again the experiment indicates that the casein particles are smaller than the chicken sarcoma virus. A number of similar experiments gave results of identical significance.

Filtration of Herpes Virus.

The herpes virus used in these filtrations was prepared by taking either a freshly glycerolated or a fresh unglycerolated brain of a rabbit dead of herpes, grinding it thoroughly in sand, taking it up in varying amounts of Ringer's solution and centrifuging for 1 or 2 hours until the supernatant fluid was moderately opalescent. The virus filtrations were done with dilutions of this material. There seems no particular reason for stating dilutions, since on centrifugation such varying quantities of brain material were thrown down that the amounts of possible virus in the supernatant fluid cannot be estimated in any manner that could have any significance.

Comparison of Casein and Herpes Virus.

Membrane M 2.—1 per cent–¼ min.–6–4 min.–30 min. 95 per cent. Divided into 2 parts simultaneously tested.

	2 cm. Hg 10 min.	2 cm. Hg 10 min.	4 cm. Hg 5 min.
M 2a	H_2O 1.25 cc.	Casein solution 0.5 cc. negative	Casein solution 0.3 cc. positive
M 2b	H_2O 1.25 cc.	Herpes 0.4 cc. Rabbit injected	Herpes 0.3 cc. Rabbit injected

Both animals negative. Control died in 5 days.

This experiment, carried out with two sections of the same membrane simultaneously tested and showing equivalent volumes of water at 2 cm. Hg in 10 min-

utes, indicates that the herpes virus was held back by a membrane which permitted the casein to pass through.

Comparison of Casein, Herpes Virus and Rous Sarcoma Virus.

In this experiment three parts of the same membrane were simultaneously tested. In making this membrane, however, the method of dipping in collodion solution was substituted for the rotating test-tube, a fact which probably accounts for the relatively greater permeability of one of these membrane sections over the others. The table shows that K 2a and K 2b let through 1.25 cc. of water at 2 cm. Hg in 5 minutes, while K 2c let through 1.5 in the same time at the same suction. It is apparent from the results that casein came through K 2a at 2 cm. Hg, and that K 2b and K 2c held back both herpes virus and the Rous sarcoma virus at the same suction. The experiment would indicate that the casein particles were smaller than either of the two viruses used, and this result was obtained in spite of the fact that K 2c may have been slightly more permeable than the other two sections.

Membrane K 2.—1 per cent–¼ min.–6–2 min.–30 min. 95 per cent. (Dipping method, which accounts for unevenness of membranes.)

	Water at 2 cm. Hg 5 min.	Filtration, 2 cm. Hg 10 min.	
	cc.		
K 2a	1.25	Casein solution 0.8 cc.	Casein positive
K 2b	1.25	Herpes virus 0.8 cc.	
K 2c	1.50	Rous sarcoma virus 1.25 cc.	

Animals inoculated from filtrate of K 2b and K 2c negative. Controls with unfiltered virus suspensions positive.

In the next experiment a freshly made collargol solution, ½ per cent in distilled water, centrifuged at 2,000 revolutions for 2 hours, was used for comparison. The diameter of the smallest collargol particles so procured is supposed to measure approximately 20 mμ. We have no means of actually measuring these particles, and are accepting the figures of other workers.

Membrane R 2.—1 per cent–¼ min.–6–5 min.–30 min. 90 per cent. Divided into four parts.

	H_2O, 5 min. 2 cm. Hg	15 min. 2 cm. Hg		H_2O, 5 min. 2 cm. Hg
	cc.			*cc.*
R 2a	1.0	Collargol	2.25 cc. +++	0.4
R 2b	0.75	Casein	1.0 cc., acid negative, precipitate +	0.15
R 2c	0.75	Herpes	1.25 cc.	0.25
R 2d	1.0	Air test.	Air bubbles begin at 6.5 cm. Hg	

The rabbit inoculated from the herpes filtrate remained negative, indicating that the herpes virus in the condition in which it existed in the suspension was larger than the collargol.

Experiments Controlling the Possible Effect of Opposite Electrical Charges on Substances to Be Filtered and on Filter Membrane, Respectively.

The following three experiments were carried out for the purpose of controlling errors in the results of filtration which could conceivably have occurred if the isoelectric point of the particles to be filtered should happen to be on the alkaline side of pH 7.2, at which all our previous experiments were done. Olitsky and Boëz stated that the isoelectric point of the virus of foot-and-mouth disease lay at about pH 8. It seemed to us likely that if foot-and-mouth disease virus were isoelectric at this reaction, the same might be true of the viruses with which we have been dealing. Were this the case, then of course casein and the other proteins, the isoelectric points of which are in the neighbourhood of pH 5, would be negatively charged at pH 7.2, and thus easily pass through a negatively charged membrane, while the viruses at pH 7.2 would be positively charged and might be held up by conditions entirely independent of size.

For this reason, in the following three experiments Rous sarcoma virus, herpes virus and staphylococcus bacteriophage were all compared by filtration, in each case through four parts of the same membrane, and both the casein and the respective substance adjusted to 7.2 in one segment and to 8.6 in another.

The virus used was a 2 per cent suspension of a freshly prepared triturate of Rous sarcoma filtered through a sand filter. The casein solution was prepared with the purified casein furnished us by Dr. Edwin Cohn.

Membrane Q 4.—1 per cent–¼ min.–6–4 min.–30 min. 90 per cent. Divided into four parts.

	Water at 2 cm. Hg 5 min.	Filtration at 2 cm. Hg 7 min.
	cc.	
Q 4a	1.25	Casein pH 7.2, 0.75 cc. + came through
Q 4b	1.25	Casein pH 8.6, 0.75 cc. +++ came through
Q 4c	1.25	Rous sarcoma virus pH 7.2, 1.5 cc.
Q 4d	1.25	Rous sarcoma virus pH 8.6, 1.5 cc.

Chicks inoculated from Q 4c and Q 4d remained negative. Control chicks inoculated with the paper pulp sand filtrate before collodion filtration were positive in 2 weeks.

Membrane Q 5.—1 per cent–½ min.–6–4 min.–30 min. 90 per cent—divided into four parts.

The herpes virus consisted of a 7 day glycerolated brain of an herpetic rabbit; 1 gm. ground in sand and suspended in 30 cc. of Ringer's solution; centrifugalized at high speed until the supernatant fluid was clear. Casein as in the preceding experiment.

	Water at 2 cm. Hg 5 min.	Filtration 2 cm. Hg 7 min.	Water at 2 cm. Hg 8 min.
	cc.		*cc.*
Q 5a	2.75	Casein at pH 7.2, 0.75 cc. + came through	0.5
Q 5b	2.75	Casein at pH 8.6, 0.75 cc. ++ came through	1.0
Q 5c	2.5	Herpes virus at pH 7.2, 0.5 cc.	1.0
Q 5d	2.5	Herpes virus at pH 8.6, 1.0 cc.	1.0

Rabbits were inoculated from filtrates of Q 5c and Q 5d and controls with unfiltered supernatant fluid of centrifugalized virus taken from material left on membrane. The results indicated that the virus had not come through.

Comparison of Bacteriophage with Casein.

Staphylococcus bacteriophage consisting of a Berkefeld filtrate of a cleared culture.

Membrane R 1.—1 per cent–¼ min.–6–4 min.–30 min. 90 per cent.

	Water at 2 cm. Hg 6 min.	Filtration 2 cm. Hg 8 min.	Water at 2 cm. Hg 6 min.
	cc.		*cc.*
R 1a	1.75	Casein pH 7.2, 0.75 cc. + came through	0.25
R 1b	1.5	Casein pH 8.6, 0.75 cc. ++ came through	0.25
R 1c	1.5	Staphylococcus bacteriophage pH 7.2, 1 cc.	0.5
R 1d	1.5	Staphylococcus bacteriophage pH 8.6, 1 cc.	0.5

The bacteriophage was tested in the following way:

Tube 1.—1 cc. 7 hour staphylococcus culture + 0.1 cc. unfiltered bacteriophage at pH 7.2 = complete clearing up overnight.

Tube 2.—1 cc. 7 hour staphylococcus culture + 0.1 cc. filtered bacteriophage at pH 7.2 = negative.

Tube 3.—1 cc. 7 hour staphylococcus culture + 0.1 cc. unfiltered bacteriophage at pH 8.6 = complete clearing.

Tube 4.—1 cc. 7 hour staphylococcus culture + 0.1 cc. filtered bacteriophage at pH 8.6 = negative.

In the last experiment tabulated we used collargol as a colloidal suspension composed presumably of particles larger than any of the proteins used. We had found that the bacteriophage and the viruses were all held up by membranes which allowed the three proteins to pass through them. Curiously enough, although our calculations from the molecular weight—taking the density as 1 — indicated that the casein molecules should have a diameter of approximately 8.5 mμ, we found by actual filtration that casein was larger than collargol, which is supposed to have a minimum diameter of 20 mμ. Moreover, whatever the pH, casein solutions are always very slightly opalescent, a fact which should indicate that many of the particles in suspension cannot be much smaller than half a wave-length of light. It must be assumed, therefore, that casein is present either in a condition of considerable swelling or as aggregates. This is a matter that we are hardly competent to discuss in detail, but we offer the observed facts in the interests of completeness.

III.

In attempting to produce membranes of increasing permeability in order eventually to obtain filters that would allow the bacteriophage and the two varieties of virus to pass through them, we found that the alcohol-ether method could not be satisfactorily used because of the friability of membranes containing less than 1 to 1½ per cent of collodion. We therefore turned to the method advised by Bechhold, Schoep and others.

This consists of impregnating filter paper with solutions of collodion in glacial acetic acid. The permeability of the membrane is entirely determined by the percentage of the collodion solution. The membranes are hung into a bath of acetic-collodion in a vacuum chamber, the collodion being run into the bath after preliminary exhaustion, and after the air has been removed from the pores of the paper, atmospheric pressure forces the collodion solution thoroughly into the interstices of the paper. The filter paper is then allowed to drip and is finally soaked in running water for 24 hours.

Such membranes were employed in the same type of filter and in virtually the same manner as were the alcohol-ether films.

For comparative filtrations with these membranes we used, in addition to the substances to be tested, casein, collargol and freshly prepared arsenic sulfide.[2]

The following table shows that 4 per cent acetic-collodion lets through crystallized egg albumen, but holds back serum albumen and the larger particles of collargol and arsenic.

4 Per Cent Acetic-Collodion Membrane.—

Membrane	H_2O, 15 min. 8 cm. Hg		15 min. 8 cm. Hg
	cc.		*cc.*
1	1.0	Egg albumen ++	0.5
2	0.75	Horse serum albumen negative	0.5
3	0.80	Collargol negative	1.0
4	1.25	As_2S_3 negative	1.5

In the next experiment a more permeable membrane was used.

2 Per Cent Acetic-Collodion Membrane.—

Membrane	H_2O, 10 min. 2 cm. Hg	Filtration 10 min. 2 cm. Hg	H_2O, 10 min. 2 cm. Hg
	cc.		*cc.*
1	2.5	Collargol 1.75 cc., ++ bright yellow	
2	2.5	As_2S_3 1.50 cc. negative	0.75
3	2.0	Phage 0.75 cc. +	
4	2.5	Rous sarcoma 1.0 cc. negative. Control positive	0.75

This 2 per cent acetic-collodion membrane let through collargol and the bacteriophage, but held back the Rous sarcoma virus and the arsenic suspension. While

[2] The collargol suspension was made by dissolving 0.2 per cent commercial collargol both in distilled water and in salt solution, centrifuging for 2 to 3 hours at approximately 2,000 R.P.M. and using the upper layers of the fluid. The arsenic trisulfide was made by passing hydrogen sulfide through a solution of As_2O_3 in water. The arsenious acid solution was saturated by boiling, was cooled to room temperature, filtered and diluted with 3 volumes of water. This is the method

the subsequent water tests on two of these membranes showed that there had been no gross leakage—indeed, a considerable obstruction,—the delicacy with which these experiments must be done, is indicated by the fact that Membranes 1 and 3 ruptured when the suction was increased to 4 and 5 cm. Hg.

In the next experiment herpes virus was compared with casein and collargol.

2 Per Cent Acetic-Collodion Membrane.—

Membrane	H_2O, 5 min., 2 cm. Hg	Filtration	H_2O, 5 min. 2 cm. Hg
	cc.		*cc.*
a	2.5	Herpes 2 per cent 0.75 cc.	0.75
b	2.5	Casein 1 cc. acid — negative, precipitate −++	0.75
c	2.5	Collargol 1.25 cc. +++ bright yellow	1.35

The control rabbit inoculated with the material over the filter membrane died in 5 days. The test rabbit inoculated with the filtered virus died with a typical syndrome of herpes at about the same time. Fresh herpes brain from a rabbit which had just died was used in the preparation of this virus, and we believe that this may have something to do with the successful filtration.

A still more permeable membrane was made and used to compare collargol, staphylococcus bacteriophage, Rous sarcoma virus and arsenic suspension.

1.5 Per Cent Acetic-Collodion Membrane.—

Membrane	H_2O, 5 min., 2 cm. Hg	5 min., 2 cm. Hg	H_2O, 5 min. 2 cm. Hg
	cc.		*cc.*
a	2.5	Collargol 2.5 cc. ++	2.5
b	3.0	Phage 2.0 cc. +	2.75
c	3.0	Rous sarcoma 2.5 cc.	2.0
d	2.5	As_2S_3 1.75 cc. negative	1.80

The chick inoculated with the filtrate of the Rous virus on the left breast, with a control inoculation of the unfiltered virus on the right breast, began to show definite tumors on both sides within 10 days. This experiment was repeated with another set of membranes, with entirely comparable results.

Combining this with previous experiments, it would be apparent that the Rous sarcoma virus was larger than collargol and smaller than colloidal arsenic.

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described by Wolfgang Ostwald, Practical colloid chemistry, New York, 4th edition, 1924, 6.

1.5 Per Cent Acetic-Collodion Membrane.—

Membrane	H_2O pH 7.2 5 min. at 3 cm. Hg	Filtration 10 min. at 4 cm. Hg	H_2O pH 7.2 5 min. 3 cm. Hg
	cc.		*cc.*
a	2	Collargol 3 cc. +++	1.5
b	2.5	As_2S_3 4 cc. negative	1.0
c	2.5	Herpes* (0.25 per cent) 1 cc.	0.75

* Control died typically in 5 days, filtrate rabbit on 8th day.

In the above experiment herpes virus apparently came through the membrane which held back colloidal arsenic. The final water tests show that no accidental leakage or other irregularity was responsible for the result.

DISCUSSION.

The determination of size by methods of ultrafiltration is of course subject to many possibilities of error and cannot be relied upon except in defining relatively gross ranges of magnitude. We believe that the technique developed by us avoids all the obvious errors and is subject to simple control in regard to the detection of leakages, and injuries to the filter membranes in the course of the experiments. The practice of measuring the flow of water in a given time under constant pressure before and after filtration of various substances not only insures against accidents of the kind mentioned, but supplies interesting information in regard to the degree to which a membrane has been obstructed by the substance filtered. The method of making membranes in sheets and dividing them into separate parts makes it possible to filter several substances simultaneously under equivalent conditions, and the accuracy with which identical conditions can be assumed to prevail is further controlled by attaching all the different segments to the same source of negative pressure and determining the flow of water per unit time both before and after the filtration.

The alcohol-ether method of making membranes on rotating tubes, especially when reenforced by sheets of Japan paper, furnishes a delicate and very elastic method of varying permeability, but these membranes cannot be made sufficiently permeable to allow anything larger than collargol to pass through them without becoming too

soft and friable for use. More permeable membranes of sufficient strength can be made by the Bechhold method with collodion dissolved in glacial acetic acid, and this was therefore the method that we employed in the final stages of our experiments.

For determination of the sizes of the pores of our membranes we hoped to be able to use the method of Bechhold, which depends upon the measurement of the pressure necessary to force air through the filter under water. A formula has been derived by him from such measurements which has also been applied by Stuart Mudd to the measurement of the pores of Berkefeld filters. We found, however, that the calculated sizes of the pores in our collodion membranes were entirely inconsistent with the probable sizes indicated by permeability for various substances, a discrepancy that we attribute to the fact that each additional cm. of pressure upon the soft filter membranes used by us considerably bulged them in a manner which inevitably modified the pore sizes, and pressures beyond 4 and 6 cm. of mercury often completely disrupted these filters. This rendered it essential to control our membranes by the flow of water under similar pressures before and after each experiment. It will be noticed that we hardly ever used more than 2 cm. of mercury for our filtrations. Poiseuille's formula, which is based upon measurements of the pressure necessary to force water through a capillary, and has been modified by Bechhold to apply to filters, was also found inapplicable in our studies.

CONCLUSIONS.

We are submitting this series of experiments as observed facts, realizing that there are so many uncertainties in this form of indirect observation that great caution must be exercised in drawing conclusions of any kind. The most serious of the possible errors involved is that the active substances which we have studied, the enzyme—the bacteriophage—and the several varieties of virus, may not be free in our suspensions, but are adsorbed to larger particles. The peculiar difficulties encountered in filtering herpes virus particularly suggest a source of error of this kind, and if we are right in assuming the intracellular position of this virus in the nervous tissue, it is more

than likely that most of the virus obtained in suspension may be closely associated with protein particles derived from the cells. Keeping all this in mind, we may, nevertheless, derive a certain amount of information from our experiments as follows:

1. The order of magnitudes of the pure proteins with which we have worked,—namely, crystallized egg albumen, crystallized serum albumen and purified casein,—follows the order of molecular weights of these substances as determined by Cohn. As far as casein is concerned, the size indicated by filtration in comparison with collargol is far greater than it should be by calculations which take a molecular weight of 192,000 as the point of departure. While one cannot be sure of the reason for this, there are many possible explanations such as considerable swelling of the casein particles, aggregation of molecules and the fact that casein is not at its isoelectric point under the conditions of filtration and surely present as a salt.

2. Trypsin, even in the certainly very impure condition in which we employed it, is but very slightly larger than serum albumen and distinctly smaller than casein. In its pure form it may well be much smaller even than our filtrations indicate, but certainly not larger. This relatively small size of trypsin may have considerable bearing upon the question of whether or not the lytic agents spoken of as "bacteriophage" are substances of the nature of enzymes, or whether they are more comparable to the filtrable virus, as supposed by d'Hérelle.

3. Herpes virus, the Rous chicken sarcoma and a staphylococcus bacteriophage were all subjected to filtration at pH 7.2 and at hydrogen ion concentrations higher than 8, which is given by Olitsky and Boëz as the isoelectric point of foot-and-mouth disease, but failed to pass membranes which, at the same pressures, were permeable for casein and collargol. The bacteriophage and the Rous sarcoma with considerable regularity passed through membranes which held back colloidal arsenic trisulfide. We have cited only a few of the experiments which were actually done, every one of the tests tabulated being merely representative of a number of others that were omitted for economy of space. The herpes virus we have had greater difficulty in filtering. We cite one experiment with a 2 per cent acetic-collodion membrane and another with a 1.5 per cent

membrane through which the herpes virus passed, the membrane being so controlled that gross leakage could be excluded. We believe that the difficulty here is very largely due to the fact that in preparing the herpes virus for filtration it cannot be separated from considerable amounts of brain material, from which, perhaps, it is not easily dissociated. This would be natural if the herpes virus were intracellularly located, as we believe it to be. This experiment and similar ones, however, incline us to believe that the herpes virus is not far different from the Rous sarcoma virus and the bacteriophage, as far as filtration through membranes is concerned. It certainly is not smaller than either of these substances and probably, as we judge from a few experiments carried out at higher pressures, is not much larger.

It may be assumed, therefore, that in the form in which we were able to procure the bacteriophage and the two varieties of virus investigated by us, they were of a magnitude larger than casein and collargol and smaller than colloidal arsenic. The weak point in drawing our conclusions is the fact that we were not in a position to measure for ourselves with any accuracy the actual sizes of collargol and arsenic trisulfide particles. Accepting the general views of Bechhold and others, however, our experiments would define the sizes of the separticular substances as larger than 20 mμ and probably smaller than 100 mμ.

The order of magnitudes of the substances measured by us would then be as follows:

Crystallized egg albumen
Crystallized serum albumen
Trypsin
Collargol
Casein
Bacteriophage, Rous sarcoma virus, herpes virus
Arsenic trisulfide

Our experiments show little agreement with the work of Levaditi and Nicolau and of Levaditi, Nicolau and Galloway. In their recent filtration tests of foot-and-mouth disease this virus is reported by them as passing through membranes that held back trypsin, indicating a size much smaller than any of the viruses measured by us.

Our results, on the other hand, are in actual measurements comparable to those of Olitsky and Boëz, not only in the fact that the viruses with which we worked correspond approximately to the size determined by them for foot-and-mouth disease, but that the percentage of collodion in membranes permeable for virus and impermeable for colloidal arsenic corresponds almost exactly to our own. This gives us confidence that the technique developed may be more easily standardized than we at first believed and that the method of ultrafiltration, owing to the great ease with which membranes of relatively standard size may be made, may have valuable applications in the investigation of bacteriological and immunological problems.

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