From the Department of Virus Research, Karolinska Institutet, Stockholm, Statens Seruminstitut, Copenhagen, Statens Bakteriologiska Laboratorium, Stockholm, and the Institute of Physical Chemistry, Uppsala.

Studies on the Sedimentation of Influenza Virus*.

By

S. Gard, P. von Magnus, A. Svedmyr, and **A. Birch-Andersen.**

With 5 figures.

Introduction.

In 1946 *von Magnus*[1] described the phenomenon of "autointerference" which was observed in mice or eggs infected with influenza virus obtained on egg passage of undiluted allantoic fluid in series. The biological aspects of this phenomenon are dealt with in a series of articles by the same author[2–5]. In a preliminary paper on the physicochemical properties of different preparations, *Gard and von Magnus*[6] concluded that the capacity of autointerference was linked to the appearance in the allantoic fluid of a component, presumably a non-infective virus variant, with a lower sedimentation rate than the typical virus, but distinct from any macromolecular material that could be recovered from normal fluid.

With the methods of purification and analysis used, four main components were detected, designated and characterized as follows.

Comp. I: s_{20} about 220 S; in normal allantoic fluids and infected fluids during the early stages of infection.

Comp. II: s_{20} about 380 S; in infected fluids obtained after inoculation of undiluted virus.

Comp. III: s_{20} about 660 S; in infected fluids, primarily after use of diluted seed virus.

Comp. IV: s_{20} about 1330 S; appearing irregularly in infected fluids.

Component I was subsequently shown to carry the inhibiting activity found in normal allantoic fluid (*Svedmyr*[7–8]). In infected fluids the total amount of sedimentable material represented by components II and III

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was largely constant and corresponded to the hemagglutination titer. On the other hand, the proportions of the two components varied characteristically, depending upon the inoculum used. Whereas, when diluted seed virus had been used, component II was absent or present in only moderate amounts, fluids obtained by inoculation of undiluted seed showed a striking preponderance of this component. In parallel with the disappearance of component III the infectivity decreased. It was concluded that component III represented the virus of the classical type, characterized by infectivity as well as capacity of hemagglutination, while component II presumably was hemagglutinating but noninfectious and capable of eliciting the phenomenon of autointerference.

Later, evidence was presented that component II was a product sui generis and not a result of secondary disintegration of active virus[9]. It was also shown that components II and III remained distinct in artificial mixtures as evidence of their individuality.

The nature and origin of component IV remained obscure. It was found in infected fluids only and was supposed to represent virus aggregates or cell debris.

Early during these experiments it became evident that the interpretation of sedimentation diagrams of the complex nature often met with in virus preparations of varying purity required a more thorough study of the technical factors involved. Consequently, it was decided to study the influence upon the sedimentation pattern of variations in the composition and concentration of the preparations, the interest being centered on the problem of identifying a particular substance by means of sedimentation analysis. This problem will be the subject of later communications. It shall only be stated here that great caution is necessary in the interpretation of results when several macromolecular components are present in the material. In this case serial runs with varying concentrations of the specimen are indispensable.

The present paper contains data collected during these studies relating to questions concerning the "incomplete" virus.

Material and methods.

Strains of virus.

A. The Copenhagen PR 8 strain (PR 8–146) was obtained in 1941 from Dr. *R. E. Shope*, Princeton N. J. and has subsequently been maintained in mouse-egg passages. Egg passages were initiated from the 80th mouse lung passage. The material used in the present experiments was derived from the 87th to 198th passages. In 1944 an independent line of this strain was started in Stockholm from the 6th mouse lung

passage. After one additional passage in mice, the Stockholm strain was maintained in allantoic passages. Some of the specimens examined were derived from the 55th to 67th egg passages of this line.

B. An egg-adapted Lee strain was used in some experiments. This strain was obtained from Dr. *C. H. Andrewes*, London. It was maintained in serial allantoic transfers and the specimens examined were derived from the 23rd to 25th egg passages.

Inoculation and titration. The preparation of inocula and the procedures employed in inoculation and harvest of the virus were in accordance with the description given by *von Magnus*[2], as was the technic for titration of infectivity and hemagglutination and determination of immunizing capacity. Only eggs of White Leghorns were used. Batches of 600 to 1500 eggs were inoculated at a time, each egg receiving 0.1 ml or 0.2 ml of the seed; the yield of allantoic fluid varied from 3 to 9 liters. Sometimes several batches of fluid from eggs inoculated with the same inoculum were pooled and processed in volumes of 10 to 12 liters.

Purification. Depending upon the purpose of the experiment one of two different procedures was employed.

a) After filtration through cotton wool the allantoic fluid was passed through a Sharples Presurtite Supercentrifuge equipped with a virus recovery bowl at a rate of flow of about 1.5 liters per hour and a speed of 50 000 r.p.m. The sediment was washed in the bowl with 1000 ml. of buffered saline and taken up in the residual 120 ml of wash fluid. The material was clarified by centrifugation at low speed and then spun in the airdriven ultracentrifuge at 27 000 r.p.m. (ca. 50 000 x g) for 20, 60 or 90 minutes. The sediments were resuspended and clarified by centrifugation "up-and-down" to 10 000 r.p.m. or at 5000 r.p.m. for 5 or 10 minutes either in the ultracentrifuge or in an ordinary angle centrifuge.

b) After filtration as above the allantoic fluid was concentrated and partially purified by means of ultrafiltration through collodion coated porcelain filters[10]. After reduction of the volume to $^1/_{20}$ to $^1/_{60}$ of the original the material was subjected to differential centrifugation in the ultracentrifuge in two cycles of 27 000 r.p.m. for 90 and 60 minutes respectively and "up-and-down" to 10 000 r.p.m. or at 5000 r.p.m. for 10 minutes.

With both procedures the final concentration by volume was usually 1000-fold. Specimens prepared according to method (*a*) usually did not contain any demonstrable quantities of the slow sedimenting "normal" components of the allantoic fluid ("200 S" and "30 S"), whereas the second procedure gave a product where both these components were present.

Diluents and wash fluids consisted of 0.1 molar phosphate buffer of p_H 7.0 or of a *phosphate-saline**. As preservatives, merthiolate in a final concentration of 1 : 10000 or penicillin and streptomycin (100 units/ml and 100 γ/ml) were added. It was attempted to maintain a temperature of about + 4° C during the whole period of preparation.

Sedimentation analysis. The procedure will be described in detail in a subsequent paper. Here it will only be pointed out that the sedimentation rates were measured by optical registration according to *Lamm*'s scale method in the *Svedberg* "equilibrium" type of centrifuge at a temperature of + 20° C and in centrifugal fields of ca. 6000 x g (= 10000 r.p.m.) or ca. 12000 x g (= 14000 r.p.m.). Unless otherwise stated, the sedimentation constants quoted in this paper were calculated from serial experiments with varying concentrations of the material. Thus the figures generally represent sedimentation constants at infinite dilution obtained through extrapolation of the concentration curves.

Viscosity was measured in an *Ostwald* Viscosimeter at + 20° C; adjusted time of out-flow for distilled water 120 sec.

Nitrogen determinations were performed according to the micro *Kjeldal*-method described by *C. Brecher*[11] using boric acid for titration of the ammonia.

Experimental.

In general, our previous observations were confirmed. In the present connection we intend to deal mainly with the components designated as II and III (see above) and include only a brief summary of further results.

The existence of the four main components was confirmed. It was previously reported that component I was absent in infected fluids at the time when virus was demonstrable. This statement has been found to be incorrect. When proper methods of purification and concentration were applied, a substance with the sedimentation characteristics of component I could always be demonstrated in infected and normal fluids alike. In specimens treated according to method (*b*) two additional normal constituents were found, one of them obviously corresponding to the "30 S" component[12], and another still slower sedimenting with s_{20} of about 4.5 S.

Occasionally, a fast sedimenting, fairly homogeneous component IV was observed. No attempts were made to identify it biologically. The strains of virus used in the present study have been found on electron

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* KH_2PO_4 27,96 g
Na_2HPO_4, $2H_2O$ 58,43 g
NaCl 48,00 g
Aq. dest. ad 10000 ml.

microscopic examination to yield a certain amount of filamentous material. Tentatively, it might be assumed that component IV is made up of such filaments.

The sedimentation pattern in serial passages of undiluted allantoic fluid virus. Serial passages with large doses of seed virus were carried out according to *von Magnus*[2]. The allantoic fluids were harvested at the time of maximal activity, concentrated and purified as described under methods. Before processing each fluid was tested for infectivity and hemagglutinating activity. On account of inactivation during purification, infectivity measurements were not suitable for the calculation of losses during purification. As the hemagglutinating activity was apparently not affected, the yield was always calculated on the basis of hemagglutinating capacity of the purified specimens. In Table I the characteristics of representative preparations of PR 8 virus under standard conditions and in the first three passages are listed.

Some of the figures of the table need special comments. The I/A ratio, i. e. the ratio of infectivity titer to hemagglutination titer, serves to characterize the virus preparation. A typical standard virus shows an I/A ratio of about 6 log units. During the first three or four passages of undiluted fluid a downward shift in this ratio takes place. On some occasions values approaching 0 have been obtained. A typical third passage virus should have an I/A ratio not exceeding 3.0 log units[2–5].

The specific activity was calculated as the logarithm of hemagglutinating units per mg. of nitrogen. The average for the whole series of PR 8 preparations was 6.0 and the deviation from the mean of anyone of the individual values was less than 0.3 i. e. within the limits of error of the methods of assay. There was, thus, no indication of significant differences in purity or true differences in hemagglutinating capacity between the different forms of virus.

There seemed to exist definite differences in viscosity between materials derived from different passages. The mean relative viscosity of solutions containing 1 mg of nitrogen per ml. was 1.25, 1.35, 1.50 and 1.70 for preparations of standard, first, second and third passages, respectively. Since, however, a considerable variation in this respect was encountered in successive preparations of similar passages, viscosity measurements *per se* proved of little value as a means of characterization, although when considered together with other findings they could serve to render the picture more complete.

Standard passages. The first series of specimens were derived from the Copenhagen PR 8 strain. When examined in the ultracentrifuge, the majority showed the presence of two components. A typical sedimentation diagram is reproduced in Fig. 1. The sedimentation constants were calculated from the data obtained by analysis of series of different

Table I.

Passage	Batch no.	Starting material				Concentrate			Conc. by volume	Yield %	Spec. activity
		Initial volume	EI 50 log.	CCA log.	I/A ratio	CCA	mg N/ml.	η			
Standard	82	9450	10.2	3.9	6.3	6.2	2.47	1.770	750	35	5.8
1st pass.	87	10980	9.7	3.8	5.9	6.7	3.96	2.579	1140	70	6.1
2nd „ ...	106	11170	8.0	3.8	4.2	6.1	2.02	2.228	1000	20	5.8
3rd „ ...	100	14260	5.8	3.3	2.5	6.0	1.48	2.225	790	65	5.8
3rd „ ...	85	8700	5.8	3.3	2.5	6.0	1.40	1.891	890	55	5.9
Standard	St. 1	4800	—	4.1	—	6.8	4.31	n. t.	1000	50	6.2
3rd pass.	88	9960	6.6	3.7	2.9	6.5	1.96	3.588	840	80	6.2
Standard	St. 2	active				6.9	4.00	n. t.	1000	80	6.3
„	St. 2	heat inactivated				6.7	4.80	n. t.	1000	50	6.0
Lee standard	79	8440	9.5	3.7	5.8	6.0	1.83	1.476	790	25	5.7
Lee 3rd pass.	81	11200	7.2	3.6	3.6	6.1	1.60	2.733	500	65	5.9

Data on batches of allantoic fluid virus used as type specimens in the present paper.

Abbreviations: EI 50 = egg infectivity 50% endpoint; CCA = chicken red cell agglutination; I/A ratio = log ratio of EI 50 and CCA; η = relative viscosity. Specific activity is calculated as CCA per mg. nitrogen; n. t. = not tested.

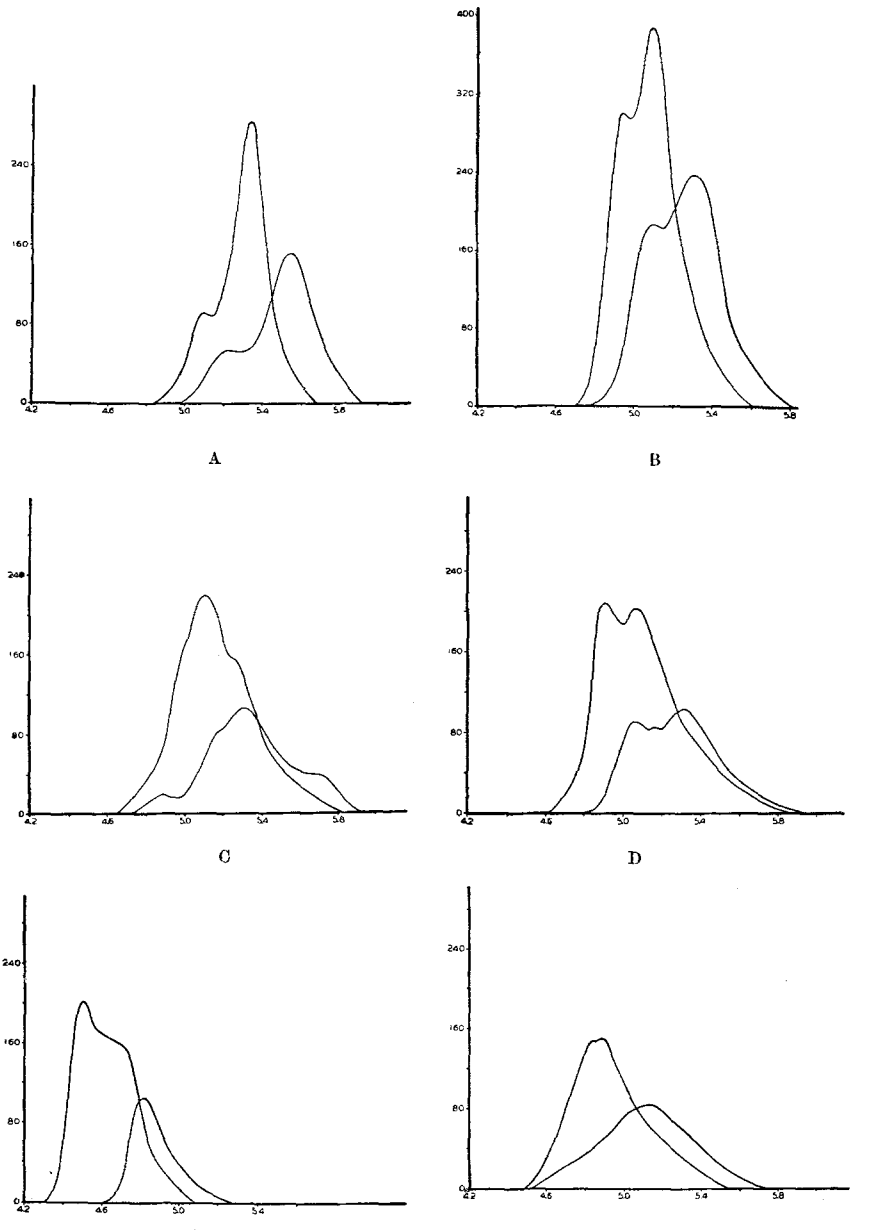


Fig. 1.

Sedimentation diagrams of purified allantoic fluid virus. Strain Pr 8 Copenhagen. Ordinate: Scale displacement. Abscissa: distance from the axis. Rotational speed (except in E): 10000 r.p.m. Exposures as indicated below.

A) Standard passage (batch 82); 0.82 mg N/ml; 30 and 40 minutes.
B) First passage (batch 87); 1.19 mg N/ml; 30 and 40 minutes.
C) Second passage (batch 106); 0.61 mg N/ml; 40 and 60 minutes.
D) Third passage (batch 100); 0.74 mg N/ml; 40 and 60 minutes.
E) Third passage (batch 85); 1.40 mg N/ml; 14000 r. p. m.; 20 and 60 minutes.
F) Same specimen; 0.70 mg N/ml; 10000 r. p. m.; 40 and 60 minutes.

concentrations. It was not always possible to obtain any reliable figures relating to the slower component. When computable, the sedimentation constant was found to be in the neighborhood of 600 S (extremes 580 and 670; mean 633).

The constant of the faster component was calculated to an average of 747 S $\pm$ 4. On two occasions, however, considerably higher values were obtained, 810 and 879, respectively. The deviation from average in the last two cases is far too great to be accounted for by chance variation. The reason for the aberration could not be definitely established. The possibility of a mixup with Lee virus could be ruled out. The possibility of temporary defects in the recording devices of the centrifuge seems very remote as both preparations were examined in a large number of different concentrations during two separate periods and each series was interpolated with several runs of other preparations giving "normal" results. A checkup on all data concerning inoculation and purification of these batches has revealed nothing differing from the routine procedure. At present we can only state the fact that these two specimens differed from the rest with regard to the rate of sedimentation and omit a discussion of the reason for this finding. The appearance of the sedimentation diagrams showed nothing to distinguish them from those obtained with other preparations of standard virus. Curve A in Fig. 1 is a tracing of exposures obtained with the scale method from one of these preparations (batch no. 82).

As seen in the diagram, the faster component is by far the predominating one. The slower component can be estimated at between 10 and 20 p.c. at most of the total detectable material. This type of curve is representative for the results obtained on analysis of batches prepared from the Copenhagen PR 8 strain. The Stockholm strain on the other hand always gave curves of the type shown in Fig. 4 and 5 A, i.e. with only traces of slow sedimenting material. This difference is probably referable to strain characteristics as it was constantly encountered also in batches of the Stockholm strain cultivated and prepared in the Copenhagen laboratory. It was thus independent of the eggs used, the conditions of cultivation and the unavoidable small differences in the purification procedures employed in the two laboratories.

The above statements refer to specimens treated according to procedure (*a*), i. e. without initial ultrafiltration (see methods). In material so prepared, the "normal" component (the hemagglutination-inhibitor) was usually not demonstrable. Occasionally traces of it could be demonstrated in centrifuge diagrams of material in high concentrations. In such cases it was always distinct from the other components and usually no interpretation difficulties ensued. On the other hand, when procedure (*b*) was applied, the "normal" component appeared in the final preparations in approximately the same concentration as found in specimens derived from normal allantoic fluid. The sedimentation pattern of such specimens seemed to differ from the (*a*) type and not seldom presented serious difficulties of interpretation. A discussion of this problem will be included in a following paper. Unless otherwise stated, diagrams and figures in the present paper all refer to observations made on specimens prepared according to procedure (*a*).

First passage. Fig. I B shows a typical diagram obtained from purified first passage material. The similarity to the standard passage is obvious in that the same two components with sedimentation constants 606 and 745 are present. However, the proportions are different, as the slower component approaches 50 p.c. of the detectable solids. Provided that the faster component represents the fully active virus and the slower the non-infectious hemagglutinin, and provided that the specific hemagglutinating activities of the two forms are the same, the I/A ratios from Table I would indicate a content of infective virus in the first passage of about 40 p.c. of that in the standard material. The actual proportions of the components in diagram I B are as 1 : 1 which must be considered as in good agreement with the assumptions just made.

A closer analysis of the diagrams from different concentrations of the material shows the 745 S component to be comparatively homogeneous as indicated by the moderate spreading of the boundary during the passage through the cell. Diffusion can obviously play no part as a spreading factor in the range of particle sizes dealt with in the present connection. Any broadening of the boundaries must, therefore, be referred to a certain inhomogeneity of the component in question. However, the shape of the peaks corresponding to the 745 S component indicates a normal and rather narrow distribution of sedimentation rates. The 606 S boundary on the other hand showed a definite tendency to spreading on prolonged sedimentation as well as on dilution of the material. The shape of the peak was similar to that in Fig. 1 D, demonstrating a distinct deviation from a normal distribution of sedimentation velocities. Two extreme values of the sedimentation constant could be calculated and found to be 545 S and 667 S respectively.

Second passage. In the centrifuge the preparation described as type specimen in table I (batch no. 106) showed one main component, displaying a certain tendency to split into two (Fig. 1 C). The sedimentation constant was calculated to 568 S. In addition, traces of slower sedimenting material were observed, probably representing small quantities of the "normal" component. Furthermore, in specimens sufficiently concentrated a faster boundary was observed. The sedimentation constant of this material was calculated to 767 S, a value which must be regarded as only approximate on account of the uncertainty in defining the position of the boundary and the limited range of concentrations available for study. It can hardly be doubted, however, that this component corresponds to the standard virus of $s_{20} = 747$ S. The proportion of this component can be estimated from the diagrams as not more than 5 p. c. of the detectable solids. According to Table I a content of active virus of about 1 p.c. should be expected.

Third passage. In specimens of this type usually no component corresponding to the standard virus could be detected. Sometimes its presence was suggested by a slight irregularity of the frontside of the sedimenting boundaries, but it was never found in quantities large enough to permit a calculation of the sedimentation constant. The amount did not seem to exceed 1 p.c. of the total detectable solids. According to Table I, less than 0.1 p.c. of the agglutinin should be expected to carry the full virus activity.

The main component was always more or less heterogeneous, often showing a tendency to split up into two or more constituents as illustrated by the curve in Fig. 1 D. Usually the slowest sedimenting part appeared to be the least inhomogeneous. This is demonstrated in Fig. 1 E and F showing the modifications of the scale diagram taking place during sedimentation and on dilution. The shape of the peak in the 20 minute exposure of the dilution 1 : 1 suggests the presence of two components. In the exposure taken after 60 minutes the faster component is recognizable only as an assymmetry of the curve. When diluted 1 : 2 the specimen shows only one rather inhomogeneous component, presumably corresponding to the slowest one. This example serves to emphasize the importance of serial analysis over a considerable range of concentrations as a means of characterization.

The inhomogeneity of the main component of third passage specimens was reflected in a considerable variation in the sedimentation constants calculated from different preparations. In some cases it was possible to calculate a maximum and a minimum value corresponding to the extremes in the diagrams, sometimes only the minimum value could be computed, and sometimes the figure obtained was of the nature of a mean. Table II contains sedimentation constants calculated from data obtained in serial

centrifugations where a sufficient number of concentrations were examined to permit a reasonably accurate determination. As shown in the table the sedimentation constants of the constituents now under discussion ranged from 430 S to 679 S.

Table II.

Passage	Strain	Batch no.	Component I	Component II			Component III
				min.	mean	max.	
Stand	K	73	—		—		747
,,	K	72	—			670	810
,,	K	86	—		580		879
,,	K	105	—	568		649	737
,,	K	12441	—		+		762
,,	S	St 1	—		—		742
1st	K	87	—	545		667	745
2nd	K	83	—	430			+
2nd	K	106	—		626		+
2nd	S	33	—			679	+
3rd	K	85	—	439		+	—
3rd	K	100	—	483		+	—
3rd	K	111	—	502		597	—
3rd	K	88	—		466		(+)
3rd	K	107	—	530		651	—
3rd	K	15120	234		501		—
4th	K	108	—		502		—
4th	K	109	—	434		+	—

Sedimentation constants (extrapolated to infinite dilution), expressed in Svedberg units. K = Copenhagen; S = Stockholm; + small amounts of sedimenting substance, probably representing the component indicated under the corresponding heading; — no traces of corresponding components.

Fig. 2 is a graphic representation of the data on component II from Table II. A general trend towards lower sedimentation constants with increasing passage number is evident. At the same time new, slow sedimenting components seem to appear with increasing frequency, as indicated in the diagram by means of different symbols.

In batch 15120, prepared according to method (*b*) a "normal" component with a sedimentation constant of 234 S was observed. A similar component was never observed with certainty in (*a*) preparations although the presence of very small quantities was sometimes probable.

The Lee strain. Similar phenomena were observed in passages of the Lee strain of influenza B virus. Fig. 3 shows sedimentation diagrams of a standard and a third passage of this strain. In both specimens two components were present but in different proportions. A successive shift in the sedimentation diagrams was observed in the course of the passages.

In the standard preparations (Fig. 3 A) the slow sedimenting component contained about 15 p.c. of the sedimenting material. In the second passage not shown in the figure the corresponding value was 20 p.c. and in the

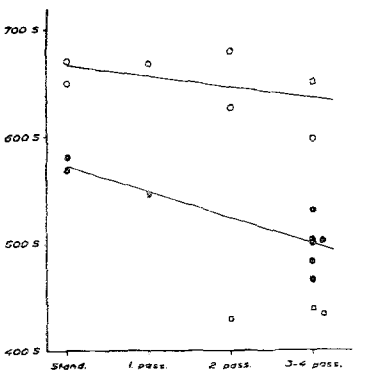
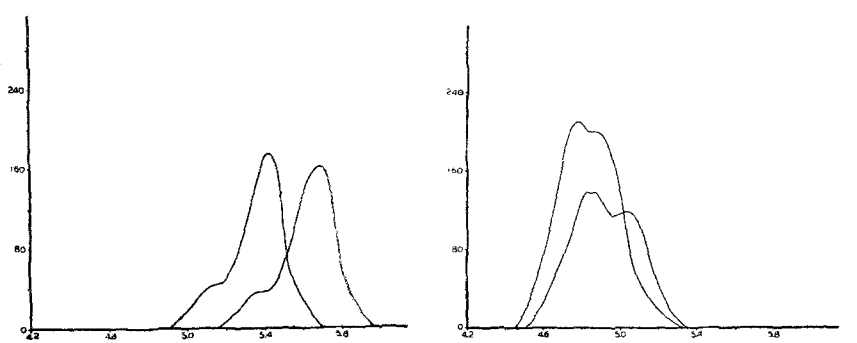


Fig. 2.

Sedimentation constants at infinite dilution of components designated as component II in passages of different orders (cf. Table II). Symbols ○ ● □ indicate possible subcomponents.

third passage, finally (Fig. 3 B), approximately 50 p.c. of the detectable solids were found in the slow component. Thus, the trend was principally the same as in PR 8 preparations, although less pronounced. The sedi-



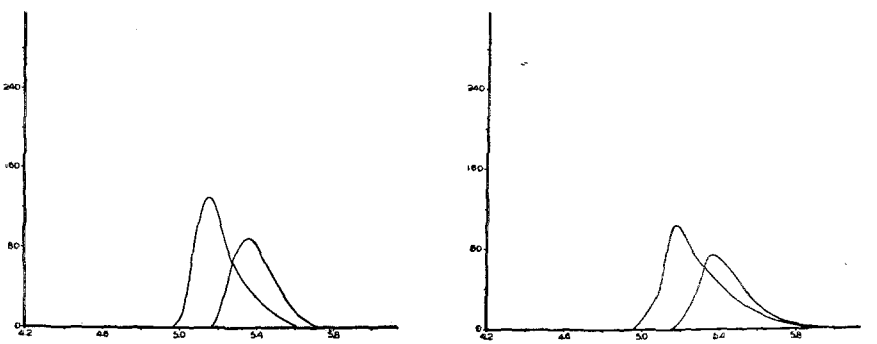
A Fig. 3. B

Sedimentation diagrams of purified Lee virus. 10000 r.p.m. Exposures 30 and 40 minutes. A) Standard passage, B) Third passage.

mentation constants calculated from serial analysis of the specimens were 894 S, 822 S and 725 S for the faster component and 644 S, 602 S and 423 S for the slower one in standard, second and third passages, respectively.

Effect of heat inactivation upon rate of sedimentation. As it has been suggested that the appearance in second and third passages of slow sedimenting components were the result of a degradation of virus from inactivation in the eggs at incubator temperature, it was considered necessary to study the effect of inactivation in vitro.

For the following experiment the Stockholm strain of PR 8 virus was used since it was known to give standard preparations practically free from slow sedimenting material. One batch of standard allantoic fluid was concentrated in the Sharples centrifuge and then divided into two parts one of which was inactivated in a water bath at + 65° C for



A Fig. 4. B

Sedimentation diagrams of PR 8 virus (batch St. 2). A) Active virus, B) Thermoinactivated virus.

15 minutes. The purification of the two portions was continued according to method (*a*). As shown in Table I the CCA and specific activities of the two purified specimen were approximately the same. Sedimentation diagrams are reproduced in Fig. 4. The two preparations appeared to be practically identical. In particular, no slow sedimenting material could be detected in any of them. The concentration of the visible component was the same within the limits of error of the method of assay. The sedimentation constants determined in single runs were 720 S and 686 S, respectively. The difference is less than 5 p.c. and within the normal variation of the method. (It should be emphasized that these values are single determinations and not obtained through extrapolation from serial centrifugations.)

Thus, there was no indication that thermal inactivation in vitro caused a disintegration of the virus particles leading to appearance of slow sedimenting components.

Analysis of allantoic fluid at different times after inoculation. In order to find out whether slow sedimenting components might be

produced by a breakdown of standard virus in the allantoic cavity a series of experiments were carried out, two of which will be quoted. In a standard passage batches of eggs were harvested at 1, 12, 18, 24, 30, 36, 44, 48 and 72 hours after inoculation. The allantoic fluids were purified in two cycles of high-low speed runs in the air-driven centrifuge and concentrated 100 times by volume. Each specimen was analysed in the ultracentrifuge. A series of fluids from a second passage harvested at 2, 6, 9, 12, 15, 18, 21, 24, 36 and 44 hours after inoculation were treated in the same way. The results of the sedimentation analyses are collected in Table III.

Table III.

Standard passage					Second passage			
incub.	comp. I	comp. II	comp. III	ratio II/III	incub.	comp. I	comp. II	comp. III
1 h	226	—	—	—	2 h	+	—	—
12 h	+	—	—		6 h	158	+	—
18 h	+	—	—		9 h	+	+	+
24 h	+	—	—		12 h	?	277	+
30 h	?	409	643	50 : 50	15 h	?	439	+
36 h	?	259	488	55 : 45	18 h	?	380	+
44 h	?	359	573		21 h	?	330	+
48 h	?	353	635	42 : 58	24 h	?	420	+
72 h	?	419	577	40 : 60	36 h	?	412	+
					44 h	?	406	+
		360 ± 63.5	583 ± 62.2				381 ± 51.6	

Sedimentation constants of allantoic fluid virus harvested after different periods after inoculation. Ratio II/III = ratio of planimetrically determined amounts of components II and III.

The standard passage in this particular experiment turned out more like a first passage, which, however, seems to be of minor importance in the present connection. Up to 24 hours after inoculation nothing could be detected in the sedimentation diagrams except small amounts of the normal component. At 30 hours a small amount of virus protein was present. Two components were clearly distinguishable with sedimentation constants of 409 S and 643 S, respectively (determined in single runs). The two components appeared in the approximate proportions 50 : 50. In all subsequent harvests two components were observed in proportions ranging from 40 : 60 to 55 : 45. The total amount of sedimentable material increased up to 44 hours and then remained largely constant.

The sedimentation constants showed a certain variation, the mean values being 360 S ($\sigma = 63.5$) and 583 S ($\sigma = 62.2$), respectively. The

large variation must be considered against the background of the following facts: (*a*) the sedimentation constants are determined in single runs and are not corrected to infinite dilution, (*b*) with the method of purification used small and varying amounts of the highly viscous normal component of the allantoic fluid will appear in the final product, influencing the sedimentation rates of the faster components. These facts taken into account, the variation seems not unduely large and in any event no difficulties were encountered in identifying the different components.

In the second passage series the presumed virus component began to appear at 6 hours after inoculation and was present in measurable amount from 12 hours onwards. It was inhomogeneous from the beginning, showing in addition to the main component small amounts of two faster ones, the sedimentation constants of which could not be determined. The relative quantities of these components did not undergo any appreciable change in the course of incubation. The mean value of the sedimentation constant of the main component was 381 S ($\sigma = 51.6$).

These experiments showed that no appreciable changes in the physicochemical properties of the sedimentable material in the allantoic fluid took place during incubation for a period of up to 72 hours. Thus, in second passages in particular the appearance of the slow sedimenting material was not preceded by release into the allantoic cavity of measurable amounts of standard virus. The first traces of sedimentable material to be detected were already of the type characteristic of hemagglutinin obtained from fluids harvested at the time of maximum activity.

Sedimentational integrity of the components observed. As each one of the components observed showed a considerable variation with regard to the rate of sedimentation and, for that reason, the interpretation of sedimentation diagrams as to the identity of any particular boundary could be subject for discussion, it was deemed advisable to examine the behaviour in the centrifuge of artificial mixtures of standard and third passage virus.

For this purpose batches St. I, standard passage of the Stockholm strain, and 88, third passage of the Copenhagen strain, were used (see Table I). Of the purified preparations dilutions were prepared containing 0.43 and 0.39 mg N per ml, respectively, and a mixture of the two at the same levels of concentration. The sedimentation diagrams of these three specimens are shown in Fig. 5. Each one of the original preparations contained a single component with sedimentation constants of 697 S and 348 S respectively (as determined in single runs). In the mixture two components are discernible with the general characteristics of each one of the ingredients. The sedimentation constants were found to be 630 S and 343 S.

Thus, the sedimentation rate of the slower component was virtually unaltered. The faster component sedimenting in the viscous solution of the third passage material should be expected to move at a lower rate than the control. The relative viscosity of a third passage solution con-

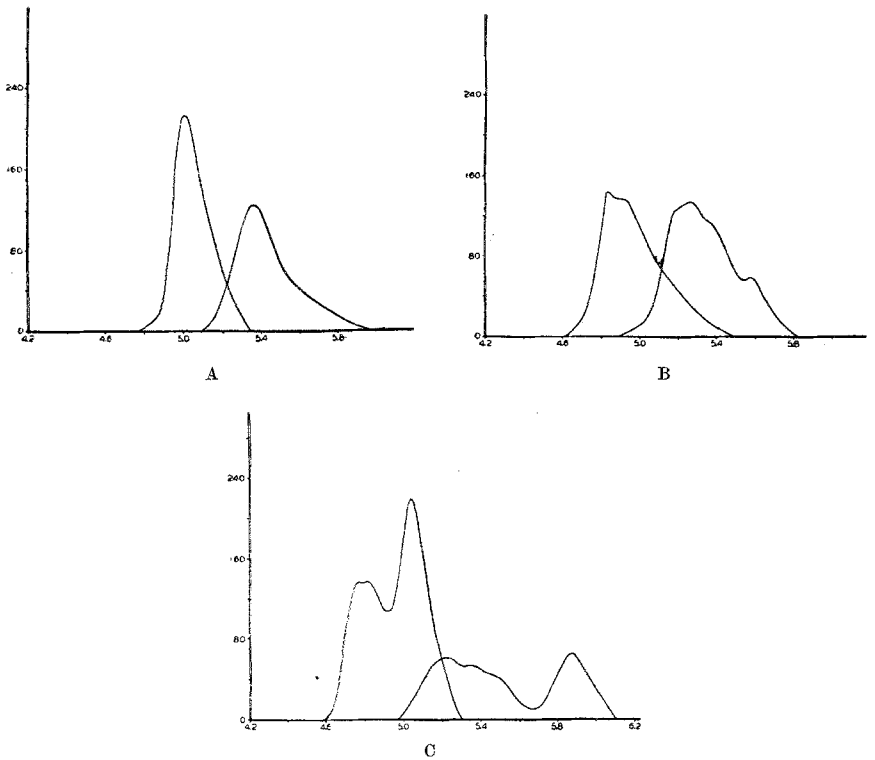


Fig. 5.

Sedimentation diagrams of PR 8 virus. 10000 r.p.m.
A) Standard passage (batch St. 1) 0.43 mg N/ml; 30 and 40 minutes.
B) Third passage (batch 88); 0.39 mg N/ml; 30 and 40 minutes.
C) Mixture of A and B; 0.43 and 0.39 mg N/ml respectively; 30 and 60 minutes.

taining 0.39 mg N per ml was found to be 1.229. The rate of sedimentation in the mixture should then be expected to correspond to a sedimentation constant of 570 S. The observed figure 630 S differs with about 10 p.c. from the expected, a difference that can well be due to chance variation. Thus there was no indication of a specific interaction in vitro between the two substances.

As a result of this experiment it can be stated that the substances obtained from standard and third passage material behaved as two

distinct compounds retaining their original sedimentation characteristics when artificially mixed.

Discussion.

During our work on the physico-chemical properties of the influenza virus produced in serial passages of undiluted allantoic fluids it was found that the sedimentation analysis of these materials, which often contained several macromolecular or particulate components, presented a number of problems of a more or less technical nature, hitherto insufficiently studied. It thus proved to be much more difficult than originally expected to produce conclusive physico-chemical evidence of the existence of two separate virus variants. However, after a comprehensive study of the sedimentation patterns under different conditions, the results of which will be presented in a subsequent paper, we feel convinced that the salient features of the earlier observations have been reproduced to a sufficient degree to permit the drawing of definite conclusions.

It must be pointed out, however, that certain irregularities have not yet been explained. For instance, it must still be left an open question, whether the conventional method of extrapolating to infinite dilution gives an even approximately correct value of the sedimentation constant. The figures mentioned in this paper are calculated according to the above method. They are presented mainly for purposes of comparison with the constants computed by other investigators, and as an illustration of the degree of reproducibility of the results. In our opinion, however, it would be unwise to attach to them such significance as to let them serve as a basis for calculations of particle size or shape.

The sedimentation rate of any one component in a complex system must be influenced by the presence of the others. Thus, differences in the relative and absolute concentrations of the different components give rise to a variation in sedimentation velocity, making it virtually impossible to identify any particular component from data obtained in a single run in the centrifuge.

According to *Lauffer*[13], [14] the variation in sedimentation rate of the virus particles should be due mainly to the viscosity of the test solution which in turn were determined by the amount of "30 S" component present. By correcting for the viscosity of the specimen the true sedimentation constant of the virus component could be obtained.

This appears to be an oversimplification of the actual facts. In normal allantoic fluid three high or macromolecular components can easily be demonstrated: *a*) one substance, possibly albumin, with a sedimentation constant of about 4.5 S, *b*) the "30 S" component, and *c*) the normal hemagglutination inhibitor with a sedimentation constant in the hundreds. These are all major components. Moreover, there may exist additional

substances in concentrations below that necessary for identification in the ultracentrifuge. In most "purified" virus preparations smaller or larger amounts of the 3 components listed above are present, the proportions depending upon the method of preparation and the degree of purification. The specific viscosities of the various normal constituents of the allantoic fluid, calculated on the basis of nitrogen content, show large differences. Furthermore, the type of relationship between concentration and relative viscosity seems to vary; in most cases being logarithmic, in some arithmetical. For these reasons it proved impossible to predict the concentration–sedimentation rate relationship in any particular preparation. In general, one would expect the *Lauffer* correction to give too high values for the purest preparations where the viscosity is mainly due to the presence of virus. This was also found to be true. On the other hand, it was often observed that correction for viscosity of the solution gave too low values in specimens where appreciable amounts of slow sedimenting components were present. The possible implications of these observations shall not be discussed in this connection. They are mentioned only to emphasize the fact that no reliable information as to the identity of any particular component can be obtained from a single centrifugation experiment. The method of choice for analysis of specimens of this character is serial centrifugations over the whole of the practicable range of concentrations.

There remains little doubt that the biological activity characteristic of the fully active influenza A virus is connected with the substance usually designated as the "700 S component". This was also borne out by the present experiments. In standard preparations close to 100 p.c. of the total nitrogen could be accounted for by a comparatively homogeneous component the sedimentation constant of which was calculated to 747 S.

Lauffer and *Stanley*[14] observed in some of their purified specimens less homogeneous components, sedimenting at lower rates. They seem to consider these substances partly as constituents of the normal allantoic fluid in the nature of impurities, partly as degradation products of the virus. In a large series of experiments we have convinced ourselves that with the method of purification mainly employed in the cases reported in this paper, at most only traces of normal allantoic fluid constituents are present in the final product, in quantities too small to be detected by the method used. Nevertheless standard preparations of the Copenhagen strain of PR 8 virus consistently contained a slow sedimenting component, usually in quantities corresponding to about 10 p.c. of the sedimentable material. In contrast to this, specimens prepared from the Stockholm strain seemed to be homogeneous. In the present connection, however, this difference seems not to be of any importance, as the two

strains appear to be similar with regard to their capacity of producing the phenomenon of autointerference as well as slow sedimenting components after serial passage of undiluted material. We are inclined to interpret the differences observed as strain characteristics, particularly since it has been found that the Stockholm substrain deviates from the mother strain in other respects as well, for instance with regard to thermostability[15].

Friedewald and *Pickels*[16] were the first to direct attention to certain discrepancies in the sedimentation rates of infectivity and hemagglutinating capacity. They concluded that their PR 8 preparations contained two components, one endowed with both qualities, the other slower sedimenting one being non-infectious but possessing hemagglutinating capacity. Like *Lauffer* and *Stanley*, these authors seem to have regarded the non-infectious material as degradation products of the active virus.

After the observation of *von Magnus*[1] that repeated passages of undiluted allantoic fluid leads to the production of non-infectious hemagglutinin, and similar phenomena described by *Hoyle*[17], by *Henle* and *Henle*[18], and by *Schlesinger*[19], it seems very probable that some type of incomplete virus can be formed either as an intermediate stage in the reproduction process or, under certain conditions, as the final result of infection. *Gard* and *von Magnus*[6] presented evidence to show that the slow sedimenting components in purified specimens consisted of such incomplete virus. These preliminary results have been confirmed and some new information added by the present experiments.

The existence and integrity of the slow sedimenting material have been proved beyond doubt. The reproducibility of the results and above all the maintainance of the individual characteristics in artificial mixtures warrant this conclusion. Therefore, it seems safe to conclude that, during serial passages of undiluted material, there is produced a substance or substances which are not present in normal allantoic fluid nor in fluid from eggs inoculated with small doses of virus. As this "component II" is the only material demonstrable in third passage specimens, it must be assumed to carry the hemagglutinating activity of such preparations. This assumption obtains further support in the good correlation between the ultracentrifugal characteristics and the biological properties of the preparations. The decrease in I/A ratio in the course of the passages is parallelled by a change in the proportions of the two main components in surprisingly good agreement with calculated values.

Inactivation of standard virus by heat or prolonged storage is not accompanied by the appearance of split products demonstrable in the ultracentrifuge. This fact alone is, of course, insufficient evidence for the assumption that component II is released as such from the cells and not an artifact produced by enzymatic break-down of virus in the allantoic

cavity. As, however, examination of the allantoic fluid in second or third passages at short intervals during the whole period of incubation showed that the appearance of component II on no occasion was preceded by production of demonstrable amounts of standard virus, the first explanation seems to be the most probable one. It is extremely unlikely that an enzymatic break-down of such rapidity should take place in second and third passages and no disintegration at all occur in standard passages. If such were the case after all, there could be but one explanation: fundamental and far-reaching differences in the properties of the virus produced under different conditions. Investigations by *von Magnus*[4] concerning the type of interference obtained with third passage virus showed that the mechanism must differ in some respects from that observed when formolized, heat inactivated, or irradiated virus is used as interfering agent. These experiments prove likewise, therefore, that component II cannot be mere inactivated standard virus. The most probable theory seems to be that component II represents an incomplete virus, a normal intermediate stage in the virus synthesis, prematurely released from the cells under the conditions prevailing in the experiments described.

As to the size and shape of the particles forming the incomplete virus, no reliable information has as yet been obtained. Filtration experiments and electron micrography have not been carried out to a sufficient extent to permit definite conclusions. The most reliable data so far are the results of the sedimentation analysis. For reasons to be discussed in another connection, the sedimentation constants found cannot serve as a basis for calculations of the particle size. It may be assumed, though, on account of the consistently lower rate of sedimentation that the particles of the incomplete virus are smaller than those of the standard virus. Furthermore, in contrast to the latter, component II showed a considerable inhomogeneity. A perusal of the sedimentation diagrams has convinced us that the variation in sedimentation constant was discontinuous rather than continuous, indicating that component II consists of a varying number of fractions of distinct properties. The data summarized in Fig. 2 seem to indicate that new fractions of ever smaller particles appear in the course of the passages.

Summary.

Sedimentation analysis of standard PR 8 influenza A virus, obtained after intra-allantoic inoculation of small doses of seed virus, showed the presence of one comparatively homogeneous main component (standard component) with a sedimentation constant of 747 S $\pm$ 4 (extrapolated to infinite dilution). In one of the two strains examined small amounts of an additional component (component II) with a lower sedimentation rate was regularly observed.

After inoculation of large doses of seed virus, particularly after serial passages of undiluted allantoic fluid, the amount of slow sedimenting material increased, while the standard component decreased proportionately, the total amount of sedimentable material remaining approximately constant. This development parallelled closely the change in biological properties of the yield.

In sedimentation experiments no interaction between the two components was observed. In artificial mixtures of the two, both ingredients retained their physico-chemical characteristics.

Under conditions when mainly component II was produced, this production was not preceded by the appearance of standard virus. In vitro inactivation of standard virus did not lead to formation of component II. It was concluded that the slow sedimenting material is produced and released as such by the cells and not the result of a break-down of preformed standard virus.

Component II showed a considerable inhomogeneity. Evidence of the existence of several fractions with distinct particle sizes was found. On serial passages a tendency to the appearance of ever slower sedimenting material was observed. The sedimentation constants ranged between 430 S and 675 S.

It is assumed that component II represents an incomplete virus, probably an intermediate stage in the normal virus synthesis, under the conditions of the present experiments prematurely released from the cells.

The same phenomenon although less pronounced was observed with a Lee strain of influenza B virus.

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