AN ULTRACENTRIFUGAL ANALYSIS OF CONCENTRATED STAPHYLOCOCCUS BACTERIOPHAGE PREPARATIONS

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PLATES 2 AND 3

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During the later stages of Northrop's study (1) of the bacteriophage for *S. albus*, the writer has had the opportunity of examining many of his preparations with an analytical ultracentrifuge arranged for ultraviolet absorption measurements according to the original method of Svedberg (2). Several things can be learned from the sedimentation diagrams of dilute protein solutions photographed in this way—(1) the rate of sedimentation of any heavy molecules that may be present, (2) the homogeneity, or uniformity with respect to size and shape, of these molecules or particles, and (3) the purity of the solution. The results of the present paper throw light upon each of these points but it is clear that many of these bacteriophage solutions have a complexity that can only be resolved by much more detailed investigation.

The instrument used in these experiments was a development of the air-driven machine already described (3). The ultraviolet light, of wave lengths between 2700Å and 2300Å, was that of a high pressure mercury arc passed through the usual chlorine and bromine filters. In all experiments the photography, which was carried out automatically (4), was such that the interval between pictures was exactly 5 minutes and the exposure 1½ seconds. Preparations of concentrated staphylococcus bacteriophage have unusually strong absorption in the ultraviolet region—for a 2 mm. thick cell the optimum concentration has been ca. 0.3 mg. per cc.

The bacteriophage solutions that have been photographed fall into two main groups—final products of the chemical purification possessing the greatest concentration of bacteriophage activity thus far
obtained, and such preparations inactivated by alkali, by chymotrypsin, and by heat. All the very active solutions gave sedimentation pictures like those in Figs. 1–5. In general these solutions contain more than one ultraviolet-absorbing constituent. They always have shown relatively sharp, rapidly sedimenting boundaries like those of Figs. 1 and 5 but there also have been present larger or smaller amounts of an absorbing material that does not sediment to a measurable extent after 1 hour in a field of 200,000 gravity and sometimes traces have been seen of a "gel" structure like that to be described for heat-inactivated solutions. The factors which determine the amount and the production of these light contaminants are as yet very incompletely understood. Experiment (1) shows that all but a trace of the bacteriophage activity is precipitated from a solution held for an hour at 40,000 gravity; this demonstrates that it cannot be a property of the light "unsedimentable" material. In view of the comparative ease with which it is centrifuged from these concentrated solutions, an association of the bacteriophage with the sharp, rapidly sedimenting boundary seems probable. Though this boundary is always the most conspicuous feature in diagrams made from the final purified solutions, the amount of the substance producing it is not simply proportional to the activity. Thus, practically inactive solutions have given photographs indistinguishable from Fig. 1; similarly the solution yielding Figs. 4 and 5 was four times as active as that of Fig. 1 though their contents of protein nitrogen were the same. Assuming that a connection really exists between the heavy boundary and the bacteriophage, this must mean that the bacteriophage molecule or particle may lose activity without a detectable alteration in shape or size.

The sedimentation constants of the heavy substance measured in different active preparations have varied from about 550 to 670 \( \times 10^{-13} \) cm. sec.\(^{-1}\) dynes\(^{-1}\). Of these the upper value is probably near the true one. There are two reasons for the lower constants that have been found. The more important one is the presence of "unsedimentable" material and of small amounts of gel that raise the viscosity of the medium through which the heavy particles are moving. An attempt was made to wash these light impurities away by throwing down the heavy substance in the quantity ultracentrifuge. This was unsuc-
cessful because some of the activity of the bacteriophage seems to be lost by one ultracentrifugal precipitation and nearly all is destroyed by repetition of the procedure. The seemingly low constants found in certain samples were due to the presence of ammonium sulfate which raised the density of the solvent slightly above unity. Because the density of the bacteriophage itself appears to be lower than that of most proteins, it takes relatively little ammonium sulfate to cause a considerable reduction in the rate of sedimentation.

Determinations of density have been possible from comparisons of the sedimentation rates in water and in quarter-saturated ammonium sulfate. Several years ago Bechhold (5) found for B. coli bacteriophage a density of 1.10 and considered this evidence for its microbial character; the mere fact that staphylococcus bacteriophage is readily sedimented from its concentrated solutions in quarter-saturated ammonium sulfate shows that its density cannot be so low. Two agreeing estimates of the density of the heavy component have been made. One preparation was made up in water and in a salt solution having a density of 1.10. In water the sedimentation constant \( s_{20,w} \) was 646 \( \times 10^{-13} \); in the salt it was \( s_{20,*} = 320 \). These rates are connected by the relation

\[
\frac{s_{20,w}}{s_{20,*}} = \frac{1 - V_{ps}}{1 - V_{pw}}
\]

where \( 1/V \) is the density of the sedimenting heavy particles. Substituting the above constants, \( V \) becomes 0.834 and \( 1/V = 1.20 \). In another experiment in which the solvent had \( \rho_s = 1.12 \), \( s_{20,*} \) was 240 (Fig. 4); the same preparation diluted with water until \( \rho_s = 1.04 \) gave \( s_{20,*} = 495 \) (Fig. 5). The density of the particles producing these boundaries calculates out to be 1.19, a value decidedly lower than the 1.33 characterizing most simple proteins.

Besides retarding the rate of sedimentation through increasing the density of the solvent, the presence of considerable amounts of ammonium sulfate leads to a pronounced diffuseness of boundary (Fig. 4). The reason for this is not yet clear.

In one or two instances a series of very faint sharp boundaries (c of Fig. 3) was observed sedimenting at about a third the rate of the heavy component. The substance responsible for these has not been
identified but there seems to be no reason for relating it intimately to the bacteriophage activity.

The molecular weight of the heavy component cannot be calculated from its sedimentation constant until the diffusion constant is known, but it is possible to establish a lower limit for this weight. According to Svedberg (6)

$$M = \frac{RTs_{20}p}{D(1 - V_p)}$$

where $M$ is the molecular weight, $R$ the gas constant, $T$ the absolute temperature, and $D$ the diffusion constant. Taking $1/V = 1.20$ and $s_{20}p = 650 \times 10^{-14}$, $M = 9.60/D$. If one examines the diffusion constants determined for very large molecules by Svedberg (6) and his students, it is evident that $D$ can scarcely be less than $0.5 \times 10^{-7}$. On this basis the heavy component of active bacteriophage solutions would have a molecular weight of at least 200 millions.

The heavy component is not split when concentrated bacteriophage solutions are inactivated by chymo-trypsin. In every instance, however, the boundaries after inactivation were more diffuse (Fig. 10). The sedimentation constants also have been low, around 550-600; but, since all these inactive preparations have contained an especially large amount of unsedimentable matter, it is impossible to say whether chymo-trypsin inactivation has led to small changes in the shape or size of the molecules of the heavy substance.

Bacteriophage solutions at pH 10 gradually become inactive over a period of several days. Photographs of such solutions have been made immediately after alkalinization and after 3 days. As can be seen from Fig. 2 the heavy boundary becomes fainter while at the same time there is an increase in the amount of unsedimentable material.

Inactivation by heating for 10 minutes at a temperature of 60°C leads to a sedimentation diagram different from any of the foregoing. The most conspicuous feature of this new diagram is an extraordinarily sharp boundary that moves outward in the cell only under the influence of intense fields. At first sight this might seem due to large molecules of a very low density but its behavior in quarter-saturated ammonium sulfate and in very dilute salt solutions conflicts with such an interpretation. Instead there are reasons for considering that it
arises through the compression of a dilute protein gel. The abnormally high viscosities of these dilute inactive solutions point in this direction but the best evidence for a gel structure is the variation in observed sedimentation constants from different preparations. They have been as low as $9 \times 10^{-18}$ and as high as $23 \times 10^{-18}$. If the protein is sedimented only part way down in the analytical cell, the preparation can be rehomogenized by mixing. When this is done, however, the new boundaries formed during a second centrifugation are ordinarily less sharp (Fig. 7) and move more rapidly than before. The remixed gel is apparently less stable than it was before ultracentrifugation because its structure often collapses and the boundary disappears during the course of a second centrifugation. Thus one preparation on its first partial sedimentation (Fig. 8) gave $s_{20w} = 10$; after remixing it yielded a boundary corresponding to $s_{20w} = 22$ which vanished after the first four or five pictures (Fig. 9). Heat-inactivated solutions commonly contain in addition to this gel an ultraviolet-absorbing substance which sediments still more slowly with a diffuse boundary ($e$ of Figs. 8 and 9) corresponding to $s_{20w} = ca. 3 \times 10^{-18}$. Its occurrence suggests that it is one of the products resulting from thermal destruction of the large particles with $s_{20w} = 650 \times 10^{-18}$.

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SUMMARY

Analytical observations have been made with the air ultracentrifuge on concentrated staphylococcus bacteriophage solutions and on these solutions inactivated by alkali, chymo-trypsin, and heat.

All active solutions contain a homogeneous heavy component that sediments with a constant of $s_{20w} = ca. 650 \times 10^{-18}$ cm. sec.$^{-1}$ dyne$^{-1}$, has an apparent density of ca. $1.20$, and a molecular weight probably not less than 200 millions. There is also present some very light ultraviolet-absorbing material which is not a carrier of bacteriophage activity. The amount of the heavy component is not strictly proportional to the bacteriophage activity so that if the activity resides in it, as appears to be the case, inactivation may occur without measurable change in molecular size and shape.

When the bacteriophage solutions are inactivated by chymo-trypsin,
the heavy component is not disrupted but the sedimenting boundaries have always been fairly diffuse. As the activity gradually disappears from alkaline solutions, the heavy component is replaced by unsedimentable material. When a solution is inactivated by heating, a dilute gel is produced which sediments with an exceptionally sharp boundary in a relatively intense centrifugal field.

BIBLIOGRAPHY
2. See for example, Svedberg, T., Colloid chemistry, New York, Chemical Catalog Co., Inc., 2nd edition, 1928.

EXPLANATION OF PLATES

PLATE 2

**Fig. 1.** A series of sedimentation photographs of an active bacteriophage preparation. The boundaries marked (b) are due to the heavy component. The meniscus (a) separates the air-bubble above from the solution below. This preparation contained less unsedimentable material (which shows itself by a greater transparency of the air-bubble) than any other that was examined; varying amounts are present in succeeding figures. Mean centrifugal field ~ 4,700 gravity.

**Fig. 2.** The sedimentation diagram of an active bacteriophage preparation after standing for 3 days (in the cold) at pH 10. Note the faintness of the boundaries due to the heavy component and the large amount of unsedimentable material. Mean field ~ 5,400 gravity.

**Fig. 3.** The sedimentation diagram of an active preparation that contained an unusually large amount of unsedimentable material. The boundaries (c) correspond to $s_{20w} = ca. 160 \times 10^{-12}$. A minute leak in the cell is indicated by the gradually widening air-bubble. Mean field = 5,500 gravity.

**Fig. 4.** The diagram of an exceptionally active preparation in ammonium sulfate solution of density 1.12. Note the diffuseness of boundary of the heavy component. Mean field = 25,000 gravity.

**Fig. 5.** The diagram of the same preparation as that of Fig. 4 in a dilute ammonium sulfate solution of density 1.04. Note the increased sharpness of boundary. Mean field = 11,000 gravity.
PLATE 3

Fig. 6. The diagram of a preparation inactivated by heating for 10 minutes at 60°C. The extraordinarily sharp boundaries of the sedimenting gel are marked (d). Mean field = 146,000 gravity.

Fig. 7. The diagram of the inactive preparation of Fig. 6 after remixing from the previous centrifugation. The boundary is less sharp than before and it settles faster. Mean field = 144,000 gravity.

Fig. 8. The diagram of another heat-inactivated preparation sedimenting in an ammonium sulfate solution of density 1.10. The diffuse boundaries (e) correspond to $s_{20w} = \text{ca.} 3 \times 10^{-13}$. Diagrams indistinguishable from this except for increased rates of sedimentation have been obtained from heat-inactivated solutions containing little ammonium sulfate. Mean field = 125,000 gravity.

Fig. 9. The diagram of the preparation of Fig. 8 after remixing. The new gel boundary (d') sediments nearly twice as fast as before and disappears after the first few pictures. Mean field = 125,000 gravity.

Fig. 10. The diagram of a preparation inactivated by chymo-trypsin. Note the comparative diffuseness of the faint heavy boundary and the large amount of unsedimentable material. Mean field = 5,400 gravity.