UNUSUAL RIBOSOME PARTICLES OCCURRING DURING SPORE GERMINATION

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ABSTRACT

WOESE, CARL R. (Yale University, New Haven, Conn.). Unusual ribosome particles occurring during spore germination. J. Bacteriol. **82**:695–701. 1961.—Spores of *Bacillus subtilis* germinated for varying amounts of time were disrupted under conditions of varying divalent ion concentration and analyzed for content of ribosomes by ultracentrifugal techniques. The picture of microsome development during germination is as follows: The spore contains only 50S and 68S particles. Early in germination, 25S and 35-8S particles increase in amount first. These particles seem to be formed in close association with the cell deoxyribonucleic acid. Their binding in the cell requires a chelating agent to release them maximally. Further, 25S and 35-8S particles are the only ones sensitive to ribonuclease and the only ones produced in the presence of chloramphenicol. The kinetics of their appearance, and their relative size, suggest them as precursors of 50S particles, which increase in amount shortly after appearance of 25S and 35S particles. It was found that 31S particles do not appear until later in germination, and their appearance signals a rise in 68S material, suggesting again the oft-reported relationship: one 31S + one 50S = one 68S. We have been unable to discern any precursors of the 31S material in these studies.

The ribosome complement extractable from the resting bacterial spore is entirely different from that extractable from the corresponding vegetative cell. In the latter case, ribosome preparations contain four major classes of particles (as defined by ultracentrifugal techniques), having sedimentation constants of 31S, 50S, 68S, and 100S. These occur in relative amounts of approximately 1:2:4:2. Resting spore extracts, on the other hand, contain only 50S and 68S particles, and these in *equal* amounts (Woese, Langridge, and Morowitz, 1960). During spore germination, each class of ribosomes increases in a characteristic manner, and by the time of the first cell division the ribosome distribution is essentially that of the log-phase vegetative cell. The differences between ribosome distributions in the spore and the vegetative cell, and the changes in the distribution occurring during germination, have been helpful in elucidating ribosomal interrelationships (Woese et al., 1960).

From previous work it appeared that the various classes of ribosomes extractable during spore germination had the same sedimentation constants as those extractable from the vegetative cell. A possible exception to this was that the 31S ribosome class extracted during germination seemed more heterodisperse than would be expected from the vegetative cell results. Attempts to investigate further this heterodispersity are the subject of the present communication. By using extraction media other than the usual divalent ion-containing ones, we have been able to distinguish clearly two new classes of ribosomes which, although they occur in negligible amounts in resting spore or vegetative cell extracts, are present as major components during the germination process. These unusual particles appear to be precursors of some of the normal ribosomes.

MATERIALS AND METHODS

The materials and methods used in this study are exactly the same as those used previously (Woese et al., 1960), with the exception of the addition of two new media for extraction of ribosomes, cited below. In brief, procedures are as follows: Spores of *Bacillus subtilis* are obtained by growth on potato agar and are stored as a dry powder. Spores are germinated in a synthetic medium which contains, among other things, glucose, glutamic acid, asparagine, and 0.005 M $MgCl_2$. In this medium the first post-germinative cell division occurs at about 200 min

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after the start of the germination process (at 37 C).

Spores are germinated for a prescribed time in this medium and are then rapidly collected by centrifugation, washed in and repelleted from one of the three media described below, ground with alumina, and resuspended in the same medium. These manipulations are all performed at approximately 4 C. Subsequently, alumina and cell debris are removed by centrifugation, and the resulting supernatant is immediately analyzed (Spinco model E analytical ultracentrifuge) for the kind and amount of ribosomes present. The ultracentrifugal analysis was done at 50,740 rev/min in a Spinco type A single-cell rotor at between 5 and 10 C, using Schleiren optics.

In previously reported work, cells were sedimented before grinding from a tris(hydroxymethyl)aminomethane (tris) buffer, pH 7.3, containing 0.01 M $MgCl_2$, and resuspended in the same medium after grinding. In the current study, this magnesium-containing medium was compared to two others: one, tris buffer containing no divalent ion; the other, containing 0.05 M sodium citrate, a chelating agent. In any one experiment the same medium was used, both for suspension of cells prior to sedimentation and grinding, and for resuspension after grinding. However, the magnesium ion concentration was adjusted to 0.01 M (or 0.015 M if 0.005 M citrate-containing buffer had been used) prior to ultracentrifugal analysis.

RESULTS AND DISCUSSION

Seven major classes of ribosomes can be extracted from *B. subtilis* in various stages of germination. Not all of these particle types are present in detectable amounts at all stages of germination, however. Table 1 gives the sedimentation constants for these particles, their corresponding molecular weights (assuming the same density, shape, and degree of hydration for all) in arbitrary units, and an indication of their occurrence in the resting spore, germinating cell, and vegetative cell. It can be seen that 25S and 35-8S ribosomes are unique in that they occur in detectable amounts only during spore germination.

TABLE 1. *Relative size and occurrence of various ribosomes extracted from cells of Bacillus subtilis during the spore-vegetative cell cycle*

S value	Relative molecular weight	Occurrence in			
		Resting spore	Early germination	Late germination	Vegetative cell
17	1.1	−	?	+	+
25	2.1	−	+	+	−
31	2.9	−	−	+	+
35-8	3.5–4.0	−	+	+	−?
50	6.0	+	+	+	+
68	9.5	+	+	+	+

The S value for the "35-8S" class is not given precisely because this sedimentation constant is not actually constant throughout germination, in contrast to the other classes of particles. During early germination its value is 35S; but as germination proceeds, this value steadily increases to 38-9S.

We reported previously that much of the cell ribonucleic acid (RNA) remained bound in the debris fraction when spores or early germinating cells were extracted in the tris-magnesium buffer. In an effort to release or characterize this debris-bound RNA further, we found that much of it is made soluble by deleting the divalent ion during extraction, even more by adding a chelating agent, citrate, to the tris buffer. Further, when RNA formed during germination is labeled with C^{14}-uracil, this labeled RNA is all released when cells are ground in the presence of the citrate-containing buffer only (*unpublished data*). Therefore, if the citrate medium does not destroy ribosomes, or destroys only certain classes of them, it seems possible to better characterize ribosomes occurring in the resting and germinating spore by using a citrate-containing buffer for extractions.

In general, however, 10^{-3} to 10^{-2} M magnesium ion is reported to be necessary to the stability in vitro of ribosomes (Tissieres et al., 1959). This is found to be true for the large ribosomes 68S (and above) of *B. subtilis* also. In contrast, small ribosomes (50S or less) of *B. subtilis* are 80 to 100% stable in tris buffer, even when it contains 0.005 M sodium citrate. Concentrations of 0.02 M or more of citrate, however, cause measurable breakdown of these smaller particles. These results in vitro then, allow us to study the effect of varying the divalent ion concentration on the extraction of the smaller ribosomes (50S or less).

When cells were extracted in the presence of the various buffers, an effect not predicted from

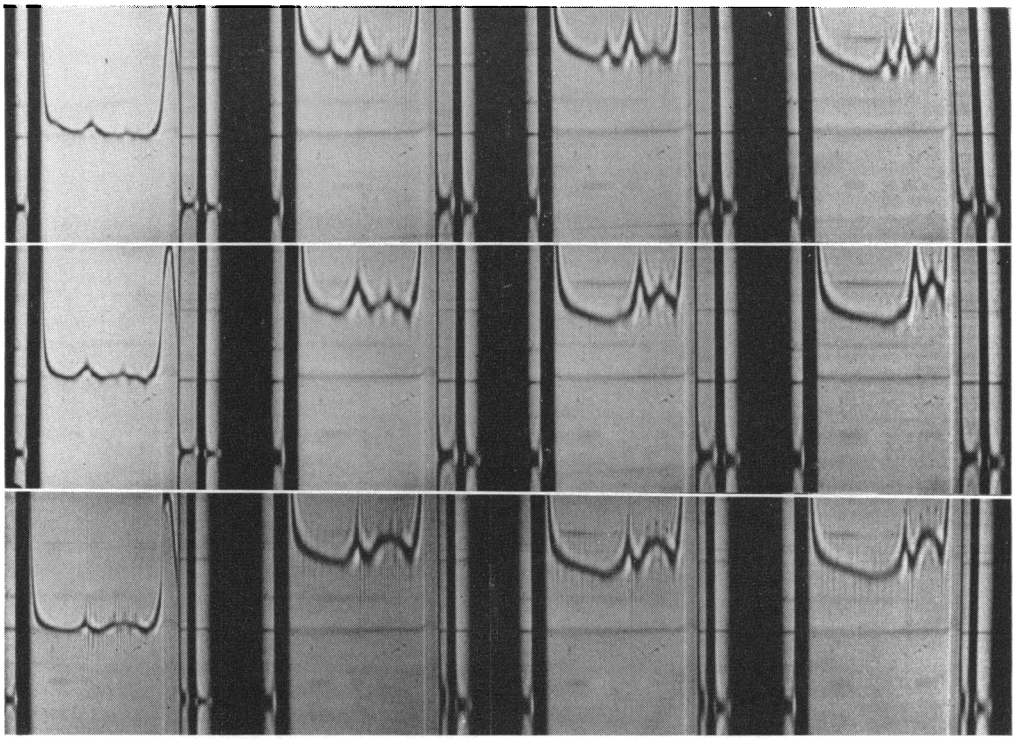

FIG. 1. *Ultracentrifuge patterns for extracts of 2-hr germinated spores. Top, extracted in tris buffer containing 0.01 M $MgCl_2$; middle, extracted in tris buffer; bottom, extracted in tris buffer containing 0.005 M citrate. In these and all subsequent figures, sedimentation is from right to left, and the time interval between pictures is 4 min.*

the above results in vitro was found. Figure 1 presents Schleiren patterns for extracts of spores germinated for 115 min and then extracted using one of the three buffers. As would be expected, 68S material occurred only in preparations made in the magnesium-containing buffer. Approximately equal amounts of 50S and 31S occur in all three cases. (As can be seen from the figure, breakdown of 68S material does not contribute much to 50S and 31S material in this case.) However, 25S and 35-8S materials are barely detectable in magnesium-containing buffer extracts, are present in greater amounts in plain tris buffer extracts, and are present in still greater amounts in citrate-containing buffer extracts.

Figure 2 shows patterns for spores extracted at various times during germination in the citrate-containing buffer. A comparison of extracts made at all stages of germination in each of the buffers reveals the following: (i) Citrate-containing buffer extracts at all stages of germination contain comparatively larger quantities of 25S and 35-8S material, but approximately the same amounts of 31S or 50S material (after correction for breakdown of 68S into 31S and 50S) as do the magnesium-containing buffer or the plain tris buffer. (ii) The resting spore contains only 50S and 68S particles in detectable and equal amounts. (In citrate-containing buffer, of course, these 68S particles do not appear.) (iii) As germination proceeds, the first changes are seen in the 25S and 35-8S particles (Fig. 2), which appear in relatively large amounts early in germination. (iv) Soon after the increase in the 25S and 35-8S classes, the 50S class begins to increase, but no detectable amount of 31S material occurs until much later (Fig. 2). There is an initial drop in the amount of 68S material (seen in magnesium-containing buffers only), and subsequent increases in 68S material appear to be coincident with the appearance of 31S material.

The initial increases in 25S and 35-8S particles, followed directly by increases in 50S particles, suggest that 25S or 35-8S particles (or both) are

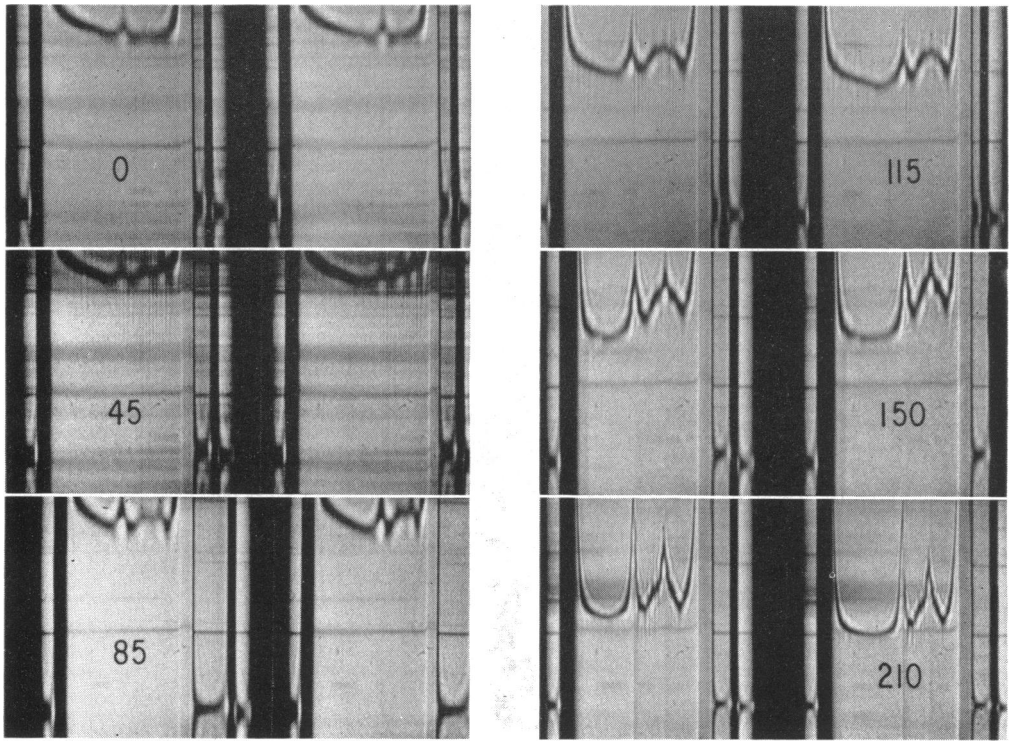

FIG. 2. *Patterns for spores germinated for minutes indicated and extracted in tris buffer containing 0.005 M citrate.*

the precursors of 50S particles. At the same time, these data make it seem unlikely that 31S material is a precursor of 50S. Further evidence favoring a precursor role for 25S and 35-8S is gained from studies in vitro. When spores are germinated for 60 min and extracted in a 0.01 M magnesium-containing buffer, the preparation consists of 50S particles almost exclusively. This preparation, treated with relatively high concentrations of citrate ion (0.03 M), will sometimes partially break down into smaller 38S particles (Fig. 3). Still higher concentrations of citrate cause 25S material to appear also. These phenomena are not completely reproducible, so no statement concerning the stoichiometry of this breakdown will be made at present. By referring again to Table 1, it can be seen that a 50S particle is equivalent in size to 6 units, whereas 25S and 38S particles are equivalent to 2 and 4 units, respectively. On this basis it is possible that a 50S particle is composed of one 25S particle plus one 35-8S particle.

It has become apparent that 25S and 35-8S particles hold a special position in the particle hierarchy. They are not detectable in either the resting spore or the mature vegetative cell, although they are present in rather large amounts during spore germination. Further, they are the first particles to increase in amount during germination, and are therefore implicated as possible precursors of other particles, probably 50S. These two classes of particles, unlike all others, are extracted to the fullest extent only in the presence of a chelating agent, citrate.

A further means of differentiating 25S and 35-8S particles from all other particles is through the action of ribonuclease. This enzyme is known to have no appreciable effect on vegetative cell ribosomes (Tissieres et al., 1959). We have found that 10 μg/ml of this enzyme have no effect on any of the particles present during spore germination except 25S and 35-8S, which are completely destroyed by 0.5 μg/ml of ribonuclease acting for less than 30 min at 4 C (Fig. 4). This suggests the possibility that these two classes of particles are composed mainly or solely of RNA. It is also possible, then, that these particles could be produced in the presence of chloramphenicol,

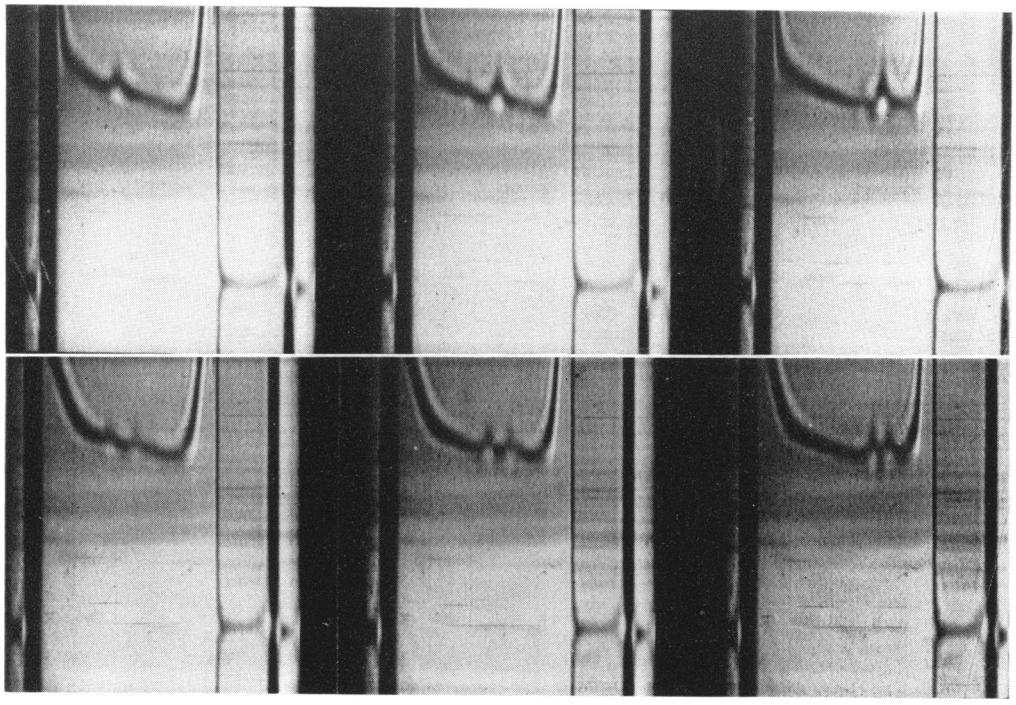

FIG. 3. *Effect of 0.03 M citrate on ribosomes in vitro, showing partial breakdown of 50S particles into 38S particles. Top, untreated; bottom, exposed to 0.03 M citrate. Time interval between pictures is 4 min.*

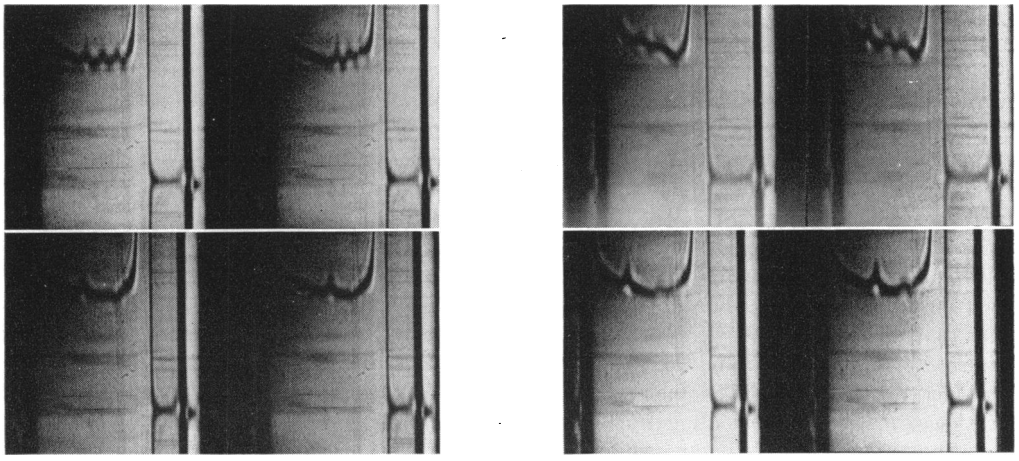

FIG. 4. *Effect of ribonuclease on ribosomes in vitro. Top, no ribonuclease; bottom, 0.5 μg/ml ribonuclease, 4 C, 30 min. Left, extract of spores germinated 1 hr, showing destruction of 25S and 35-8S material only by the enzyme. Right, extract of spores germinated 1.5 hr, showing in addition the presence of 31S material originally occluded by the 25S and 35-8S material, but becoming manifest following destruction of 25S and 35-8S by the enzyme.*

an inhibitor of protein but not of nucleic acid synthesis.

Figure 5 presents results of an experiment in which spores were germinated for 100 min in the presence of chloramphenicol (100 mg/ml) (Chloramphenicol used was a gift of the Parke-Davis Company). Although there was no increase in the amount of 50S material over that occurring in

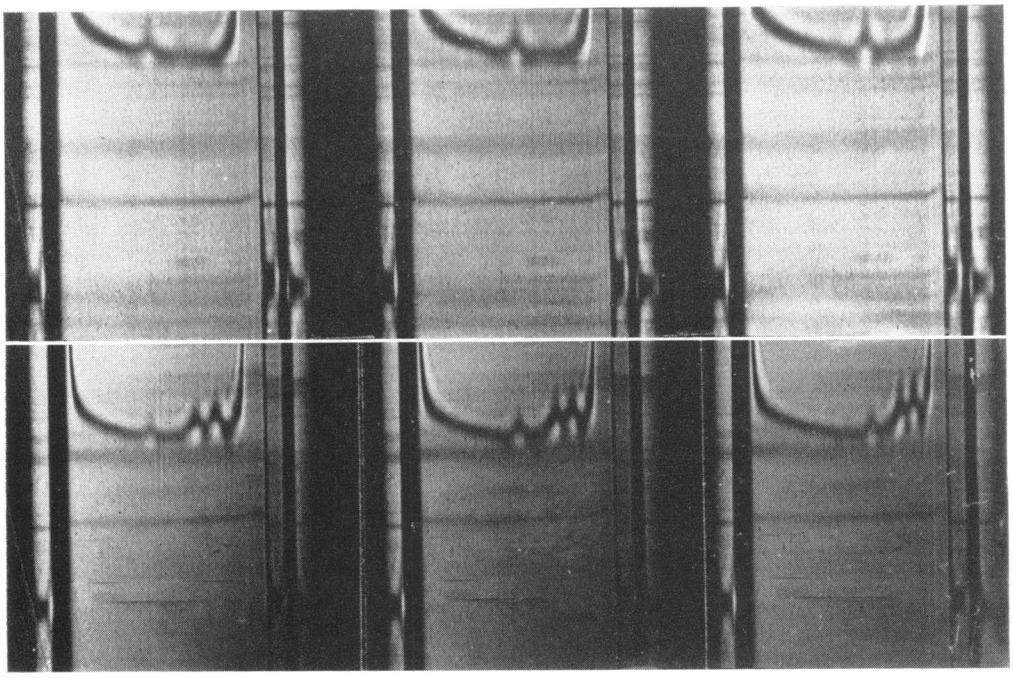

FIG. 5. *Extracts of spores germinated in the presence of chloramphenicol. Top, 0-min germination; bottom, 105-min germination. When corrections are made for Schleiren bar angle, the amount of 50S is the sa inme both cases. Extracts were made in tris buffer containing 0.005 M citrate.*

the spore state, there were essentially normal increases in the amounts of 25S and 35S material. The situation in this case is analogous to that occurring in *Escherichia coli* treated with chloramphenicol and extracted in a 0.01 M magnesium-containing buffer (Nomura and Watson, 1959). The 25S and 35S particles observed in both normal and chloramphenicol-treated *B. subtilis* germinating cultures would correspond, then, to the 24S and 31S particles observed in the inhibited cultures of *E. coli*. For *E. coli*, it was shown that these particles were composed of about 75% ribonucleic acid and 25% protein.

The fact that 25S and 35-8S material is not released maximally until cells are extracted with a chelating agent suggests the possibility that these particles may be bound specifically to other cell components. It has been shown (Ezekiel, 1961) that the ribonucleic acid formed in the presence of chloramphenicol in *Bacillus megaterium* is confined to the nuclear fraction of the cell, though not bound specifically to deoxyribonucleic acid (DNA).

In the light of these results, we feel that either or both 25S and 35-8S particles are perhaps synthesized in close association to cell DNA. Subsequently, they add protein and polymerize to produce the usual 50S particle, which then goes on to combine with a 31S particle (origin as yet unknown) to produce the 68S particle.

It is interesting to contrast the characteristics of the unusual ribosomes studied here (i.e., 25S and 35S) to those of another recently defined cellular RNA fraction, the so-called "messenger" RNA (Gros et al., 1961). In the former case, the particles in question are composed mainly or completely of RNA, are characterized by a 25S or 35S sedimentation constant, are probably formed in close association with cellular DNA, occur in amounts too small to be detected in vegetative cells, and probably combine to form the 50S particles. In the latter case, the particles are composed of RNA, are characterized by a 16S (Brenner, Jacob, and Meselson, 1961) or 8S (Gros et al., 1961) sedimentation constant, are presumably formed by cellular DNA, occur in amounts too small to be detected by standard ultracentrifugal techniques, and form a part of the 68S particles which synthesize protein. Although it is apparent that the two types of

particles are not identical, it seems possible that they may have parallel roles in the cell; namely, both are conceivably intermediaries in the process by which information stored in cellular DNA is utilized.

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