mole of each of these compounds, a molecular weight of 137,000 can be calculated using the protein content of the most active aminase preparations. This compares with a molecular weight of 140,000 calculated from the sedimentation velocity and the diffusion constant as roughly estimated by boundary spreading.

The mutual enhancement by XMP, PP_i, and psicofuranine of one another's attachment to the aminase might be the consequence of bond formation among themselves as well as with the aminase. However, failure to detect adjuncts of psicofuranine and either XMP or PP_i in ethanol-water extracts of the inhibited complex indicates that such bonds, if they are formed, could not be particularly stable. An alternative explanation might be that these compounds cause a cooperative distortion of the aminase which improves its ability to bind each of them. This latter explanation is particularly attractive since it can also be invoked in answer to the question of how binding of psicofuranine, XMP, and PP_i causes inhibition of the aminase's action. However, such configurational changes remain to be demonstrated.

References

Bergmann, F. H. (1962), *Methods Enzymol. 5*, 108.

Bratton, A. C., and Marshall, E. K., Jr. (1939), *J. Biol. Chem. 128*, 537.

Gerhart, J. C., and Pardee, A. B. (1962), *J. Biol. Chem. 237*, 891.

Kuramitsu, H. K., and Moyed, H. S. (1964), *Biochim. Biophys. Acta 65*, 504.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Monod, J., Changeux, J. P., and Jacob, F. (1963), *J. Mol. Biol. 6*, 306.

Moyed, H. S. (1961), *Cold Spring Harbor Symp. Quant. Biol. 26*, 323.

Moyed, H. S., and Magasanik, B. (1957), *J. Biol. Chem. 226*, 351.

Slechta, L. (1960a), *Biochem. Pharmacol. 5*, 96.

Slechta, L. (1960b), *Biochem. Biophys. Res. Commun. 3*, 596.

Udaka, S., and Moyed, H. S. (1963), *J. Biol. Chem. 238*, 2797.

Warburg, O., and Christian, W. (1941), *Biochem. Z. 310*, 384.

Participation of Ribosomes in the Biosynthesis of Gramicidins and Tyrocidines*

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The role of ribosomes in the biosynthesis of gramicidins, tyrocidines, and protein by cell-free preparations of *Bacillus brevis* has been investigated. With extracts of the Dubos strain of this organism, prepared under a variety of conditions, the major ribosomal component had a sedimentation coefficient ($s^\circ_{20,w}$) of 50 S. This particle, separated on sucrose gradients, was active in promoting incorporation of isotopic amino acids into both groups of polypeptides and protein. A small 32 S peak was also consistently observed, which participated only in peptide formation. Ribosomes sedimenting in the region of 70 and 100 S were detected in minor amounts under certain circumstances. Under conditions of low Mg^{2+} and high buffer concentration, two small subunits with sedimentation coefficients of approximately 18 and 27 S appeared on the sucrose gradient. Newly synthesized peptide remained bound to both these particles, while [^{14}C]protein was associated only with the larger subunit. The smaller particle had approximately the same total peptide-synthesizing activity as did the 32 S ribosome. Neither subunit was active alone in protein formation, but a recombination of the particles restored protein and peptide synthesis to their original levels, presumably by the reconstitution of the 50 S ribosome. A cellular fraction sedimenting at 40,000 $\times$ *g* was found capable of accepting the polypeptides newly synthesized by the ribosomes. Experiments with another strain of *B. brevis*, which synthesized gramicidin S, indicated the existence of the usual 30-50-70 S ribosomal pattern. When extracts were prepared in 0.1 M Tris and 5×10^{-3} M Mg^{2+}, the ribosomes appeared in the 30 and 50 S form, with biosynthetic activity residing in the 50 S unit. It was concluded that the 70 S ribosome was probably the physiological form in the *B. brevis* organism.

Ribosomes are well known to serve as the site of protein synthesis in bacterial, plant, and mammalian systems. Until recently, however, there had been no demonstration of the participation of ribosomes in the biogenesis of naturally occurring polypeptides. In fact, the synthesis of uridine nucleotide peptide (Strominger, 1962), and glutamyl polypeptide (Leonard and Housewright, 1963) seems to require only soluble enzymes.

Last year Uemura *et al.* (1963) described a cell-free system from *Bacillus brevis* which catalyzed the net synthesis of the gramicidin and tyrocidine polypeptides, as well as the incorporation of [^{14}C]amino acids into protein. An absolute requirement for ribosomes by both processes was demonstrated. Subsequent work (Okuda *et al.*, 1964a,b), has indicated a close relation-

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ship between the processes of polypeptide formation and protein biosynthesis. The similarities included the participation of s-RNA and inhibition of labeled amino acid incorporation by puromycin and chloramphenicol. The major discernible difference between the two processes involved the mode of utilization of D-amino acids in peptide formation.

The work to be reported here is primarily concerned with elucidating the role of ribosomes in the cell-free synthesis of gramicidins and tyrocidines, as compared to protein formation. Inasmuch as the ribonucleoprotein particles of *B. brevis* were found to differ in certain respects from those of other sources, some physical studies have also been included In addition, experiments were performed to distinguish between the biosynthetic activity of the ribosomes, and an apparent acceptor role of a lower-speed sediment, separated from *B. brevis* sonicates.

Experimental Procedures

Cultures.—Cells of *B. brevis* (ATCC 8185, Dubos strain BG) were grown in stationary cultures (Okuda *et al.*, 1963), using 500-ml quantities of 2% tryptone–1% yeast extract–mineral salt medium, in 3-liter Fernbach flasks. Except where otherwise indicated, this strain of the organism was used throughout the research. The cells were harvested after approximately 40 hours, near the end of the log phase (optical density reading 0.48–0.52 in a Klett colorimeter, using a 540-mμ filter). The yields were about 4 g of wet cell paste per flask.

B. brevis ATCC 9999, which produces gramicidin S, was grown with shaking on a 1% tryptone–1% beef extract–0.5% NaCl medium adjusted to *p*H 7.2 with NaOH, as described by Okuda *et al.* (1960).

E. coli strain B was cultivated at 37° with vigorous aeration, on 1% tryptone broth containing 0.5% KCl and 5×10^{-4} M $CaCl_2$, and harvested in the mid-log phase. One liter of culture yielded 1–2 g of cell paste.

Preparation of Cell Fractions.—The method was essentially that described by Uemura *et al.* (1963). All steps were carried out at 0–4°. *B. brevis* cells were washed three times with 5×10^{-3} M $MgCl_2$, and then suspended in 5 volumes of 0.1 M Tris-HCl buffer (*p*H 7.5) containing 5×10^{-3} M $MgCl_2$ and 0.01 M glutathione. The suspension was subjected to sonic disruption for 15 minutes at 60 w and 20 kc. Intact cells and cell debris were removed by centrifuging for 15 minutes at 10,000 × *g*. The supernatant phase was centrifuged, first for 30 minutes at 40,000 × *g*, and then for 1 hour at 140,000 × *g*. In some cases an intermediate centrifugation was performed for 1 hour at 70,000 × *g*. The pellets derived from centrifugation at the higher speeds were suspended in a small volume of the 0.1 M Tris buffer (usually containing 5×10^{-3} M Mg^{2+}), using a manually operated glass-Teflon homogenizer. In some cases this procedure was modified by using 0.01 M Tris-HCl buffer (*p*H 7.4), containing 0.01 M magnesium acetate for washing; while 0.01 M glutathione was included during sonication.

For certain experiments, alumina grinding was substituted for sonication. The cells were ground in the cold with a mortar and pestle for 3–5 minutes, using 3 parts by weight of alumina powder, and the usual quantity of 0.1 M Tris buffer (containing 5×10^{-3} M $MgCl_2$). Glutathione was omitted in this procedure. *E. coli* extracts were prepared by the methods described, except that the sonication time was reduced to 10 minutes.

Chemical Analysis.—Both the 40,000 × *g* and the 140,000 × *g* (ribosomal) pellets described were analyzed for protein, RNA, DNA, and chloroform-extractable material. Total solids were determined by drying a portion of the particle suspension to constant weight at 110°, and then subtracting the weight of an equal volume of buffer, dried in the same manner. Protein was determined both by the method of Lowry *et al.* (1951), and by trichloroacetic acid treatment. In the latter method, protein was precipitated by cold 5% trichloroacetic acid, and then treated with hot (90°) 5% trichloroacetic acid for 15 minutes, followed by washing with 95% ethanol, absolute ethanol, and ethanol-ether (3:1, v/v). Last, the protein was dried at 110° and weighed. RNA and DNA were determined by the orcinol and the diphenylamine methods, respectively (Schneider, 1957). Peptidic plus lipoidal materials were extracted by chloroform (from aliquots of the original suspension), dried, and weighed.

Ultracentrifugal Analysis.—Analyses were performed with a Spinco Model E analytical ultracentrifuge equipped with schlieren optics. In all cases an AN-D rotor was used, containing a cell(s) with a 1.2-cm light path. Timing was begun upon reaching a speed of 56,100 rpm, which required about 6 minutes. All runs were made at 24° in 0.1 M Tris buffer (unless otherwise specified) containing $MgCl_2$. In most cases the observed sedimentation coefficients were corrected to water at 20°, and expressed in Svedberg units. For the purpose of these calculations an average ribosomal partial specific volume of 0.65 was assumed (Petermann and Hamilton, 1961). All slopes and zero intercepts were determined by the method of least squares. The S values were in all cases extrapolated to zero concentration.

Viscosities were measured at 24.0 ± 0.05° using a (size 50) Ostwald viscometer, with a flow time for water of 343.1 ± 0.5 seconds at the above temperature. A 100-ml pycnometer was used for density measurements. In both cases deionized water was the standard employed.

Amino Acid Incorporation into Peptides and Protein.—The incorporation of isotopic amino acids into gramicidins, tyrocidines, and protein was assayed by the method of Uemura *et al.* (1963), except that the reaction was stopped by the addition of 0.4 ml of 50% trichloroacetic acid. In some cases the 40,000 × *g*, 70,000 × *g*, and 140,000 × *g* particulate fractions were reisolated (by differential centrifugation) at the end of the incubation period. In these instances the reaction was halted by chilling the system to 0°.

Sucrose-Gradient Analysis.—Linear sucrose gradients were prepared (Britten and Roberts, 1960) by mixing 3% and 20% (w/v) sucrose solutions, containing the specified Tris and magnesium concentrations, in 0.13 × 5.1–cm cellulose tubes. A ribosomal suspension (0.3 ml) (approximately 10 mg/ml) in 3% sucrose, containing the same buffer and magnesium concentration as the gradient, was carefully layered above the gradient phase. Centrifugation was then performed in a Spinco Model L ultracentrifuge for 2 hours at 28,000 rpm, using a SW 39 rotor. After centrifugation, the bottom of the tube was carefully punctured, and two-drop samples were collected.

Following gradient fractionation of ribosomes derived from prior ^{14}C labeling experiments, the individual fractions were diluted to 3 ml and the optical density at 260 mμ was measured. Then 1 ml of 140,000 × *g* supernatant was added (as a carrier), followed by 0.4 ml of 50% trichloroacetic acid. Last, [^{14}C]protein and polypeptides were separated and their radioactivities were determined in the usual way.

When the biosynthetic activity of freshly prepared (unlabeled) ribosomes was to be measured, the sucrose gradients were run in duplicate. One set of two-

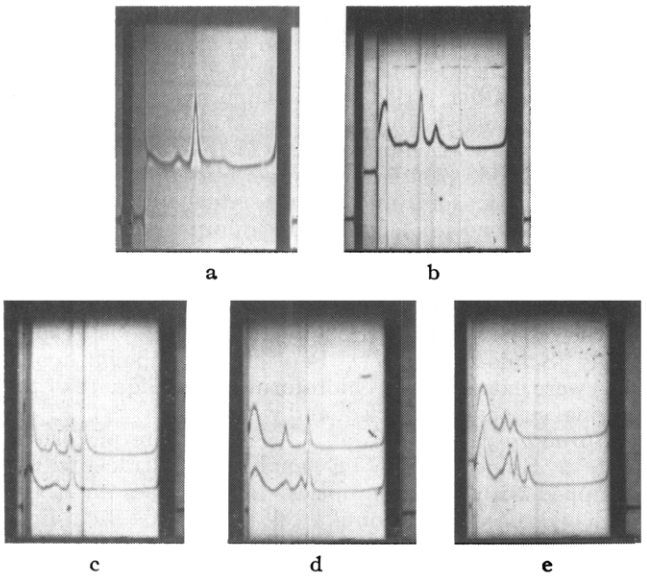

FIG. 1.—Sedimentation behavior of *B. brevis* ribosomes compared to those of *E. coli*. (a) *B. brevis* ribosomes prepared in 0.1 M Tris and 5×10^{-3} M Mg^{2+}; (b) *B. brevis* crude extract prepared in 0.01 M Tris and 0.01 M Mg^{2+}; (c) *B. brevis* (lower) and *E. coli* (upper) crude extracts prepared as in (b), after dialysis against 0.01 M Tris and 1×10^{-3} M Mg^{2+}; (d) *B. brevis* (lower) and *E. coli* (upper) crude extracts prepared as in (b), after dialysis against 0.01 M Tris and 1×10^{-4} M Mg^{2+}; (e) *B. brevis* (lower) and *E. coli* (upper) crude extracts prepared as in (a), after dialysis against 0.1 M Tris and 5×10^{-5} M Mg^{2+}. The photographs were taken 4.86, 4.52, 4.76, 7.37, and 6.55 minutes, respectively, after reaching a speed of 56,100 rpm.

drop fractions was assayed directly, using the 140,000 $\times g$ supernatant–reaction mixture system (containing a [^{14}C]amino acid); while the other set was diluted for OD measurement.

RESULTS

Composition of the Particulate Fractions.—In preparing *B. brevis* ribosomes, it was discovered that centrifugation of the sonic extract for 15 minutes at 10,000 $\times g$ was insufficient to remove completely the cell debris. Consequently a second centrifugation was performed for 30 minutes at 40,000 $\times g$, prior to removing ribosomes. The 40,000 $\times g$ pellet thus obtained consisted of a dark-brown pigmented material, with about twice the bulk of the subsequent ribosomal fraction. This pellet probably contained considerable quantities of cell-membrane fragments. The same material was observed in extracts made by either sonication or alumina grinding. Sedimentation for 1 hour at 140,000 $\times g$ yielded a transparent, amber-colored, gelatinous pellet which gave a rather turbid suspension upon dispersal in buffer solution. *E. coli* preparations, on the other hand, yielded only a relatively small quantity of 40,000 $\times g$ sediment by either method of cell rupture, and the corresponding ribosomal fraction contained less pigment and gave a clearer suspension in buffer.

Table I shows the composition of the 40,000 $\times g$ and the 140,000 $\times g$ sediments. It may be seen that there was good agreement between the two methods of protein analysis. DNA was below the level of detection in both fractions. The principal component of the 40,000 $\times g$ pellet was protein. A relatively small amount of RNA was found in this fraction, and could represent contamination by ribosomes. Semiquantitative analysis by the cellulose-charcoal method (Okuda *et al.*, 1963) revealed that a considerable part of the substances extracted by organic solvents represented gramicidins and tyrocidines. By contrast, the 140,000 $\times g$ sediment was composed almost entirely of RNA and protein in a ratio of about 1:1.

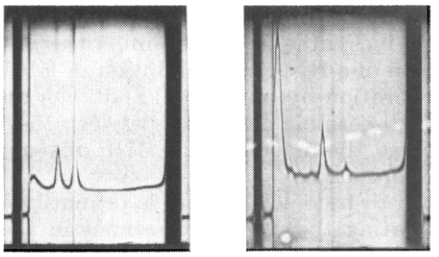

FIG. 2.—Sedimentation behavior of *B. brevis* ATCC 9999 ribosomes. (a) Ribosomes prepared in 0.1 M Tris and 5×10^{-3} M Mg^{2+}; (b) crude extract prepared in 0.01 M Tris and 0.01 M Mg^{2+}. The photographs were taken 4.60 and 2.59 minutes, respectively, after reaching a speed of 56,100 rpm.

TABLE I
MAJOR COMPONENTS OF TWO PARTICULATE FRACTIONS OF *B. brevis*[a]

Constituent	40,000 $\times g$ Sediment	140,000 $\times g$ Sediment
Protein		
Lowry method	80.0	50.2
Trichloroacetic acid precipitation	81.5	49.7
RNA	4.7	47.5
Chloroform-extractable material	11.4	1.7
Total	97	99

[a] All values are expressed as per cent of total solids (dry wt).

The ultraviolet-absorption spectra of *B. brevis* ribosomes (140,000 $\times g$ sediment) closely resembled that of *E. coli*, with a maximum and minimum at 258 and 238 mμ, respectively. The extinction coefficient of a 1% solution of *B. brevis* ribosomes was about 100 at 260 mμ, based on dry weight. This value is somewhat lower than that reported for *E. coli* (approximately 150).

Sedimentation Behavior of Ribosomes.—*B. brevis* ribosomes prepared in the standard manner (see methods) showed one principal component with a corrected sedimentation coefficient of 49.8 S, in addition to small amounts of slower- and faster-moving components (Fig. 1a). The slower particle, with a sedimentation coefficient of 32.2 S, which was regularly observed, represented about 10% of the total sedimentable material (as measured by areas under the peaks). The faster components (sedimenting in the range 60–100 S) were never present in large quantity, and were completely absent in some preparations. This same pattern has also been observed in crude extracts prepared by either sonication or alumina grinding, and then clarified by a preliminary centrifugation at 6000 $\times g$ to remove unbroken cells.

B. brevis cells were normally harvested in the late log phase, since peptide production was greatest at this stage (Okuda *et al.*, 1963). However, it may be mentioned that ribosomes prepared from cells taken from the mid-log phase (23 hours; OD 0.37) exhibited a sedimentation pattern virtually identical to that shown in Figure 1a.

Figure 1b shows the pattern exhibited by a *B. brevis* crude extract prepared according to Tissières *et al.* (1959) (alumina grinding, 0.01 M Tris–0.01 M Mg acetate). In addition to the usual 32 and 50 S particles, significant proportions of particles sedimenting in the range 70–100 S were observed. The 100 S peak was particularly sharp. In this preparation the two fastest peaks accounted for about 40% of the total ribosomes. By contrast, *E. coli* ribosomes were virtually all in the 100 S form, under these conditions.

Figure 1c gives the sedimentation pattern of a crude extract of *B. brevis* prepared as above, which had been dialyzed for 4 hours against 0.01 M Tris containing 1×10^{-3} M magnesium acetate. A comparable *E. coli* extract was treated in the same manner, and placed in the wedge cell as a control. It was found that the *B. brevis* particles moving faster than 50 S disappeared, and that the 32 S peak was rather diffuse. By comparison, *E. coli* ribosomes were largely in the 70 S form, with some 50 and 30 S subunits present. The results of dialyzing both extracts against Tris buffer containing 1×10^{-4} M Mg^{2+} are shown in Figure 1d. It may be seen that the *E. coli* ribosomes were completely dissociated into their subunits. In the *B. brevis* preparation, in addition to the usual 32 S and 50 S components, a new peak appeared with a sedimentation coefficient of 40–45 S (estimated relative to the other two peaks). This new particle apparently originated at the expense of the 50 S ribosome. The same pattern was also observed in *B. brevis* extracts dialyzed against 1×10^{-5} M Mg^{2+}.

Large amounts (more than about 10%) of 32 S particles were never observed with the Dubos variety of *B. brevis*, not even in crude extracts immediately after preparation. Likewise, only minor quantities of 70 and 100 S ribosomes were found.[1] The proportions of the faster peaks appeared to decrease with the age of the preparation. However, the 32 and 50 S pattern (in a ratio of about 1:9) was very stable, and remained unchanged on storing for at least 3 weeks, or after incubation at 37° for 4 hours. When *B. brevis* ribosomes were prepared in the presence of 0.1 M Tris and 5×10^{-3} M $MgCl_2$, followed by 4-hour dialysis against 0.1 M Tris and 5×10^{-5} M $MgCl_2$, the pattern was markedly different (Fig. 1e, lower half). The 50 S peak (fastest-moving component) was greatly reduced in size, and at least two slower-moving fractions appeared. The ratio of these peaks (although somewhat variable) was about 1.5:1. The slowest-moving component (apart from the large soluble 4 S fraction) was partly resolved into two peaks upon prolonged centrifugation. *E. coli* ribosomes also appeared to dissociate into small particles on short-term dialysis under these conditions (Fig. 1e, upper half), but upon dialysis overnight only a 4–5 S peak was observed. These small *E. coli* particles were not further examined.

Crude sonic extracts (0.1 M Tris, 5×10^{-3} M Mg^{2+}) of the ATCC 9999 strain of *B. brevis*, which produces gramicidin S, showed two peaks corresponding to 30 and 50 S ribosomes (Fig. 2a). In contrast to the particles found in the Dubos extracts, the ratio between the two components (1:2) resembled that of the 30 and 50 S subunits of *E. coli*. With 0.01 M Tris and 0.01 M Mg^{2+}, the 30 and 50 S peaks were virtually absent and the predominant particles sedimented in the region of 70 and 100 S (Fig. 2b).

Investigation of the physical properties of the smaller *B. brevis* particles has proved very difficult, owing to their instability and to a decrease in their sedimentation coefficients with increasing times of dialysis. One preparation, which had been dialyzed overnight in the cold, gave corrected values of 18 and 27 S. When the dialysis was performed at room temperature, 260 mμ–absorbing material appeared very rapidly in the external solution, suggesting the activation of a ribonuclease by the high buffer and low Mg^{2+} concentrations.

[1] The corrected sedimentation coefficients for these faster particles were not determined, but they appeared to sediment at approximately the same rate as the 70 and 100 S *E. coli* ribosomes.

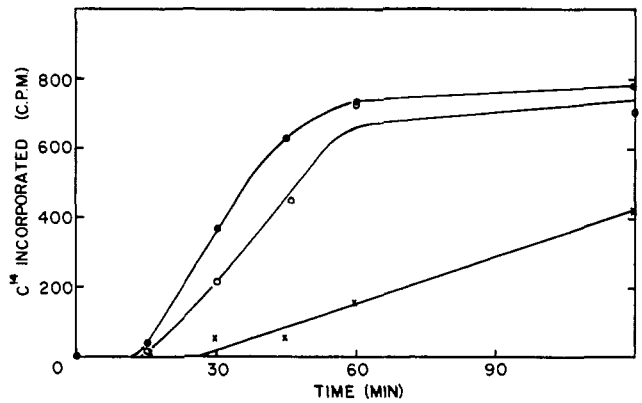

FIG. 3.—Rates of incorporation of DL-[1-^{14}C]leucine into tyrothricin of particulate fractions in standard systems. After incubation for the indicated time intervals, each reaction mixture was cooled to 0°. The fractions were immediately separated by differential centrifugation. ●—●, 70,000 × *g* sediment; 0—0, 140,000 × *g* sediment; X—X, 40,000 × *g* sediment.

Activities of Ribosomes and of 40,000 × g Sediment.—It was reported (Uemura, *et al.*, 1963) that the biosynthesis of gramicidins, tyrocidines, and protein in the *B. brevis* cell-free system required a particulate fraction sedimenting between 10,000 and 140,000 × *g*. However, it was obvious that the ribosomes prepared by this method were contaminated by cell debris, probably including cell membrane fragments. The data shown in Table II compare the biosynthetic activity of three particulate fractions. In a control assay (expt 1), the three fractions were recombined in approximately the proportions in which they occurred in the original extract. Good incorporation of [^{14}C]leucine into both polypeptide and protein was obtained. Experiment 2 shows that the 40,000 × *g* precipitate was itself virtually inactive in either peptide or protein synthesis. The 70,000 × *g* (expt 3) and the 140,000 × *g* (expt 4) sediments were each about half as active in both biosynthetic processes as was the combination in experiment 1. The gramicidin-[^{14}C]tyrocidine ratio did not vary significantly in experiments 1, 3, and 4. When the 40,000 × *g* pellet was combined with either of the higher-speed fractions (expts 5 and 6), there was a significant increase in [^{14}C]amino acid incorporation into peptide, and only a small rise in labeling of protein. The combination of 70,000 and 140,000 × *g* fractions (expt 7) restored peptide and protein synthesis to the original level.

It is noteworthy that cell extracts prepared by alumina grinding had only one-fourth the activity for polypeptide synthesis as did sonicates. Amino acid incorporation into protein was the same by the two methods. Hence only sonication was used in connection with biosynthetic experiments.

Figure 3 shows the rates of [^{14}C]leucine incorporation into tyrothricin, with each of the three particulate fractions. It may be seen that the process was rapid for the two ribosomal preparations. There was a 15-minute lag period, and after about 60 minutes the rate appeared to level off. Incorporation of isotope

TABLE II

ACTIVITIES OF PARTICULATE FRACTIONS IN POLYPEPTIDE AND PROTEIN BIOSYNTHESIS[a]

Expt	Particulate Fractions Employed	[^{14}C]Leucine Incorporated (cpm)			
		Tyrothricin	Gramicidins	Tyrocidines	Protein
1	40,000 × *g* + 70,000 × *g* + 140,000 × *g*	5300	1070	4150	3970
2	40,000 × *g*	140			460
3	70,000 × *g*	2880	730	2150	1560
4	140,000 × *g*	2910	835	2175	2080
5	40,000 × *g* + 70,000 × *g*	4380			2630
6	40,000 × *g* + 140,000 × *g*	4130			2260
7	70,000 × *g* + 140,000 × *g*	5310			3950

[a] Particulate material derived from 1 ml of sonicate was assayed under standard conditions in the presence of 140,000 × *g* supernatant solution and 0.5 μc of DL-[1-^{14}C]leucine. The 70,000 × *g* pellet had a composition similar to that of the 140,000 × *g* ribosomes (Table I), but with a slightly lower RNA content.

TABLE III

DISTRIBUTION OF LABELED PEPTIDE AND PROTEIN AMONG PARTICULATE FRACTIONS AFTER INCUBATION IN THE PRESENCE OF ISOTOPIC AMINO ACID[a]

Expt	Particulate Fraction		[^{14}C]Leucine Incorporated (cpm)			
			Tyrothricin		Protein	
	Added Initially	Added after 2 Hours	40,000 × *g* Fraction	Supernatant	40,000 × *g* Fraction	Supernatant
1	70,000 × *g*		1590[b]		1370	
4	140,000 × *g*		1520[b]		1700	
3	70,000 × *g*	40,000 × *g*	780[b]	640	20	1670
4	140,000 × *g*	40,000 × *g*	760[b]	780	160	1680

[a] The conditions employed were those of Table I. In expts 1 and 2 the time of incubation was 2 hours, and subsequently the entire reaction mixture was subjected to analysis. In expts 3 and 4 the incubations were terminated at the end of 3 hours. After chilling to 0°, the coarser particles were sedimented (30 minutes at 40,000 × *g*). Analyses were then performed separately on this fraction, and on the supernatant phase (containing ribosomes and soluble substances). [b] Gramicidins accounted for 31–37%, and tyrocidines 63–69%, of the ^{14}C incorporated into tyrothricin.

TABLE IV

EFFECT OF CERTAIN CONDITIONS ON THE TRANSFER OF LABELED PEPTIDES FROM RIBOSOMES TO 40,000 × *g* PARTICLES[a]

Expt	Conditions Employed	[^{14}C]Leucine Incorporated (cpm)			
		Ribosomal + Supernatant Fractions		40,000 × *g* Fraction	
		Protein	Tyrothricin	Protein	Tyrothricin
1	Standard conditions, with usual 140,000 × *g* supernatant	1600	1080	90	1060
2	140,000 × *g* supernatant replaced by 0.05 M, *p*H 7.4, Tris buffer	1820	1700	280	180
3	140,000 × *g* supernatant heated 5 minutes at 100° before use	1630	1210	180	620
4	Omitted ATP, phosphoenolpyruvate, and pyruvate kinase from standard system	1860	1060	76	980

[a] The ribosomes, representing 70,000 plus 140,000 × *g* particles, were labeled with isotopic leucine on a large scale in a preliminary 2-hour incubation under standard conditions. After sedimentation and washing with Tris buffer (*p*H 7.4), portions of the radioactive ribosome preparation, equivalent to the amount present in 1 ml of original sonicate, were employed in each experiment, together with nonlabeled 40,000 × *g* particles. The experiments were terminated after 1 hour of incubation, and the indicated fractions were separated and analyzed.

into the 40,000 × *g* fraction did not begin until after 30 minutes of incubation. However, the labeling continued at a slow linear rate for at least 2 hours.

One possible interpretation of the results in Table II and Figure 3 is that a transfer of newly synthesized tyrothricin from the 70,000 and 140,000 × *g* fractions (both of which behaved similarly) to the 40,000 × *g* material occurred. Table III presents data in support of this hypothesis. Experiments 1 and 2 of this table provide a measure of the preliminary labeling of peptide and protein with the higher-speed fractions. The subsequent redistribution of radioactivity in the system, following the addition of 40,000 × *g* particles, is shown in experiments 3 and 4. It may be seen that the total [^{14}C]leucine appearing in either peptide or in protein did not change significantly, and that approximately 50% of the prelabeled tyrothrycin became associated with the 40,000 × *g* fraction. However, very little [^{14}C]protein appeared in the 40,000 × *g* pellet in experiments 3 and 4).

In order to further investigate this apparent transfer of newly formed peptide, additional experiments were performed with prelabeled ribosomes (Table IV). When incubations were conducted under standard conditions (Expt 1) the radioactive peptides were distributed equally between ribosomes and larger particles, while the [^{14}C]protein remained in the ribosomal-supernatant phase. Upon replacement of the 140,000 × *g*

supernatant by Tris buffer (Expt 2), relatively little isotope was recovered in polypeptides of the 40,000 × *g* pellet. This observation suggested that the peptide transfer required a soluble component. Experiment 3 demonstrates that this soluble factor was at least partially heat-labile. Omission of ATP and the ATP-generating system led to no significant decrease in the amount of tyrothricin associated with the heavier particles. In all instances, the labeled gramicidin-to-tyrocidine ratio of the various fractions was constant (approximately 1:3). In no case was there a significant transfer of [^{14}C]protein from ribosomes to the 40,000 × *g* fraction.

Optimum Mg^{2+} Concentration for Peptide and Protein Synthesis.—It was previously shown (Uemura *et al.*, 1963) that the *B. brevis* cell-free system was most active in both gramicidin and tyrocidine synthesis when prepared in 5×10^{-3} M $MgCl_2$. Because of the importance of Mg^{2+} in stabilizing ribosomal structure, it was desirable to determine the optimum Mg^{2+} concentration for the synthesis of both peptide and protein by a second method. Figure 4 shows the biosynthetic activity of the 40,000 × *g* supernatant, after dialysis against buffer containing different concentrations of Mg^{2+}. The curves for incorporation of [^{14}C]amino acid into both peptide and protein had rather sharp peaks at 5×10^{-3} M Mg^{2+}. Above 5×10^{-2} M Mg^{2+}, protein precipitated from the reaction mixture during incubation. Labeling of protein was virtually abolished at high and at low Mg^{2+} concentrations, while peptide biosynthesis was about 20% of the optimal value under these conditions.

Biosynthetic Activity of Ribosomes Separated on Sucrose Gradients Containing High Mg^{2+} Concentrations.—The existence of 50 and 32 S ribosomal species and the apparent lack of the 70 and 100 S particles under the conditions of optimal peptide and protein synthetic activity raised the question of the relationship of these particles to the two biosynthetic processes in the Dubos strain of *B. brevis*. One approach to this problem was to incubate the ribosomes with the 140,000 × *g* supernatant and in the presence of an isotopic amino acid, under standard conditions. After incubation the labeled ribosomes were sedimented and applied to a sucrose gradient containing 0.1 M Tris and 5×10^{-3} M Mg^{2+}. Following gradient centrifugation, each fraction was analyzed for radioactive peptide and protein, and its ultraviolet absorption was measured. Figure 5 shows the results of such an experiment. Under these conditions only two peaks were observed, corresponding to the 50 and 32 S components in the analytical ultracentrifuge. No 70 or 100 S peaks appeared. It is noteworthy that newly synthesized protein was associated only with the 50 S ribosomes. [^{14}C]Peptide, on the other hand, appeared in both the 50 and 32 S peaks. It may be mentioned, in this connection, that Uemura *et al.* (1963) found radioactive peptides to be wholly bound to the (crude) ribosomal fraction of *B. brevis*, while about half the labeled protein was released into solution.

While the 32 S ribosome accounted for only about 10% of the total 260 mμ absorption, this particle contained approximately 20% of the total [^{14}C]tyrothricin of Figure 5. High-voltage electrophoresis of the latter fraction revealed that the proportions of labeled gramicidins and tyrocidines were identical in the 50 and 32 S peaks. Leucine, which was the isotopic amino acid used in this experiment, is known to be a constituent of both these classes of polypeptides.

A second procedure, designed to investigate the biosynthetic properties of *B. brevis* ribosomes, involved their prior separation on a sucrose gradient, followed by the assay of each component for activity. The results of such an experiment with [^{14}C]alanine are shown in Figure 6. This amino acid occurs in the gramicidins, but is absent from the tyrocidines. The results were very similar to those of Figure 5, except that the degree of radioactive amino acid incorporation into peptides was lower.

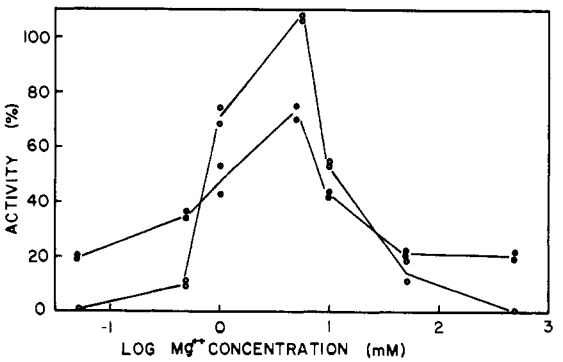

FIG. 4.—Effect of dialyzing the ribosomal-supernatant system (40,000 × *g* supernatant) against various concentrations of Mg^{2+} on subsequent biosynthetic activity. DL-[^{14}C]Leucine was the isotopic amino acid used. ●—●, ^{14}C incorporated into protein; 0—0, ^{14}C incorporated into peptide; both expressed as the percentage of the activity of the undialyzed system, assayed at 5×10^{-3} M Mg^{2+}.

In view of the unique character of the foregoing results, it seemed desirable to provide additional confirmatory data. Accordingly, the experiment of Figure 6 was repeated, using labeled DL-aspartic acid (found only in tyrocidines) and DL-leucine, in separate experiments. In each case, the results were comparable to those of Figures 5 and 6. In both of the foregoing types of experiments only radioactive peptides (gramicidins and tyrocidines) were associated with the 32 S ribosome, while both labeled peptide and protein were either attached to, or synthesized by, the 50 S ribosome.

It was of interest to determine whether 30 and 50 S ribosomes from another variety of *B. brevis* could also function independently. Consequently, ribosomes from the ATCC 9999 strain were separated on a sucrose gradient prepared in the usual way, and the biosynthetic properties of the individual fractions were assayed. The results in Figure 7 clearly indicate that the 50 S ribosome possessed activity for both polypeptide and protein synthesis. The 30 S particle, however, although present in considerable amount, had very little peptide-synthesizing ability.

Biosynthetic Properties of Ribosomes Separated on Sucrose Gradients Containing Low Concentrations of Mg^{2+}.—The *B. brevis* ribosomal subunits (smaller than 50 S) were much more readily observed on sucrose gradients than in simple buffer solutions. With buffers it was necessary to dialyze the preparation in order to witness dissociation. However, ribosomes in sucrose containing 0.1 M Tris and 5×10^{-5} M Mg readily yielded subunits. It was of interest to examine the properties of these smaller particles. For this purpose, ribosomes were labeled under standard conditions with [^{14}C]leucine as in Figure 5, and then centrifuged on a sucrose gradient containing 5×10^{-5} M $MgCl_2$ (Fig. 8). It may be seen that the 50 S ribosomes were not completely dissociated (in this particular experiment), and in agreement with previous observations this residual 50 S peak contained both labeled peptide and protein. Similarly, radioactivity was associated with the peptide and protein of the 27 S ribosomal particle. However the 18 S subunit contained only labeled peptide. The sedimentation values assigned to these two small particles are somewhat arbitrary (based on the sedimentation coefficients exhibited by preparations dialyzed over-

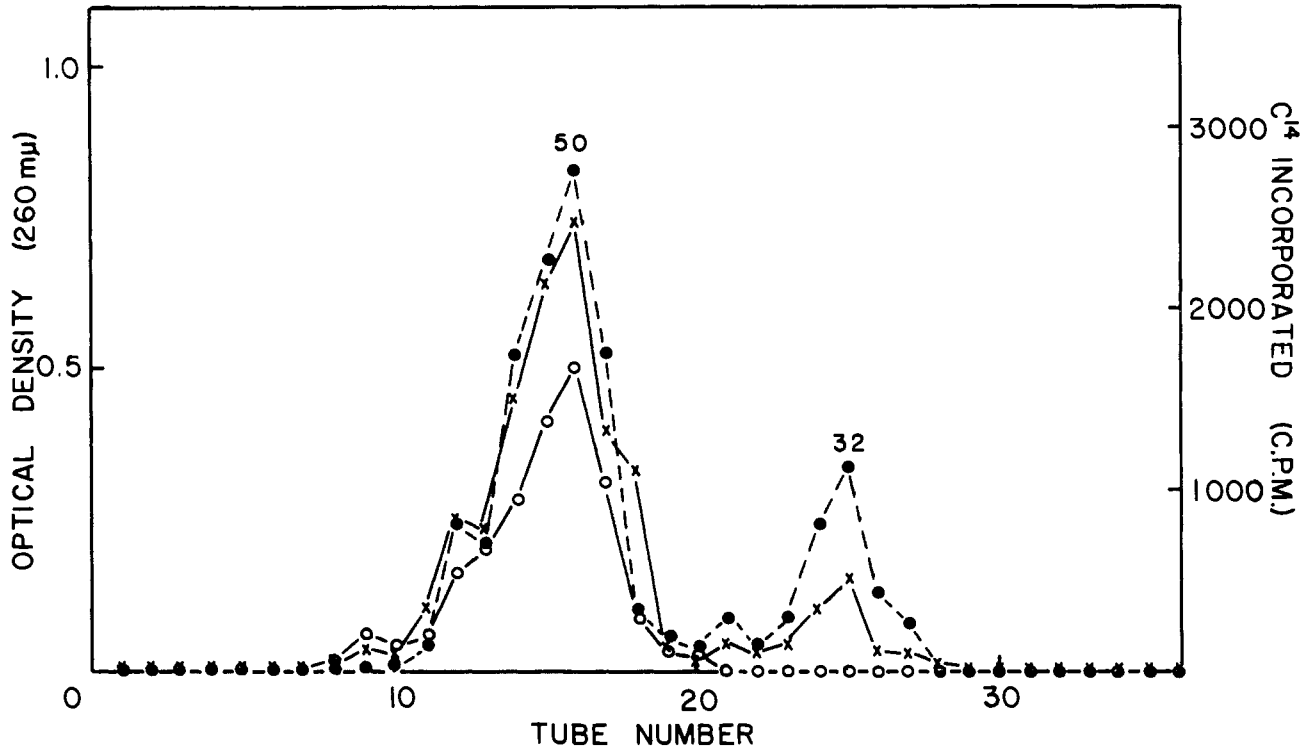

FIG. 5.—Sucrose-gradient analysis of prelabeled ribosomes in 5×10^{-3} M Mg^{2+}. DL-[1-^{14}C]Leucine was present in the standard reaction mixture at 5×10^{-3} M Mg^{2+}. X—X, OD at 260 mu; O—O, ^{14}C incorporated into protein; ●—●, ^{14}C incorporated into tyrothricin.

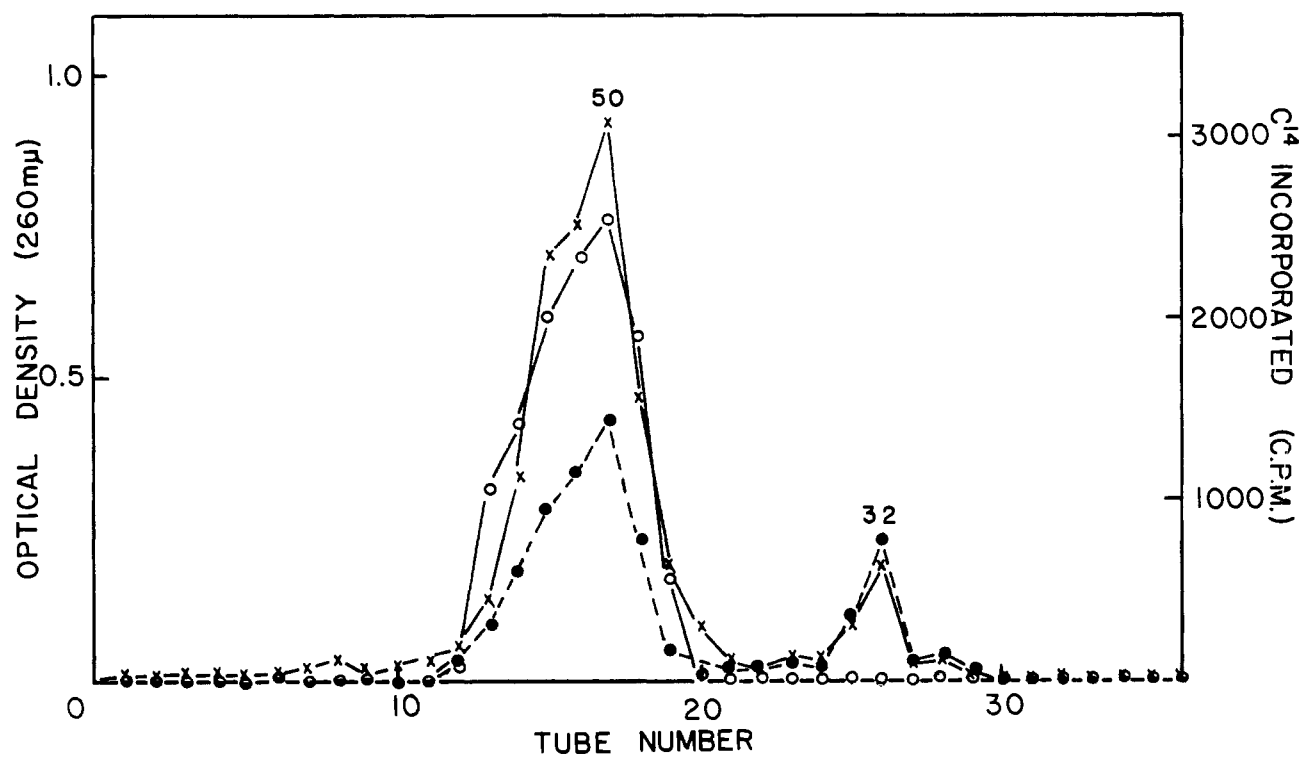

FIG. 6.—Assay of protein- and peptide-synthesizing activity of individual ribosomal fractions, following sucrose-gradient centrifugation in the presence of 5×10^{-3} M Mg^{2+}. DL-[1-^{14}C]Alanine was employed in the incubation. The symbols are the same as in Fig.5.

night in buffer solution), and are included only for reference purposes. The observation that the 27 S particle invariably sediments farther on the gradient than the 32 S particle in high Mg^{2+} suggests that the assigned S values are somewhat in error.

To confirm these rather novel findings, the same experiment was repeated using isotopic alanine (Fig. 9). This time the dissociation of the larger ribosomes was virtually complete, and the labeled peptides (gramicidins) were distributed approximately evenly between the 27 and 18 S subunits. Again [^{14}C]protein appeared only in the 27 S component.

Reference has been made to the observation (Fig. 4) that about 20% of the biosynthetic activity for peptide

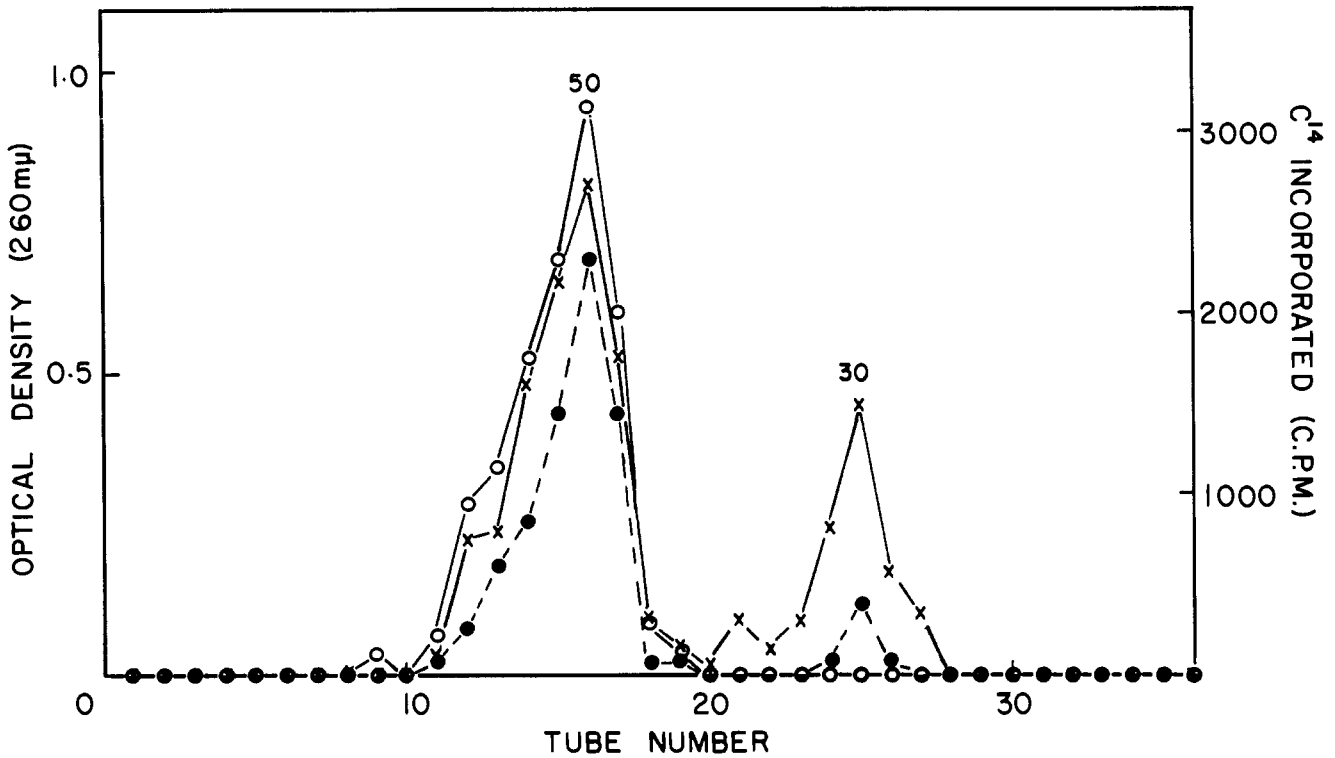

FIG. 7.—Assay of protein- and peptide-synthesizing activity of individual *B. brevis* ATCC 9999 ribosomal fractions, following sucrose-gradient centrifugation in the presence of 5×10^{-3} M Mg^{2+}. DL-[^{14}C]Leucine was employed in the incubation. The symbols are the same as in Fig. 5.

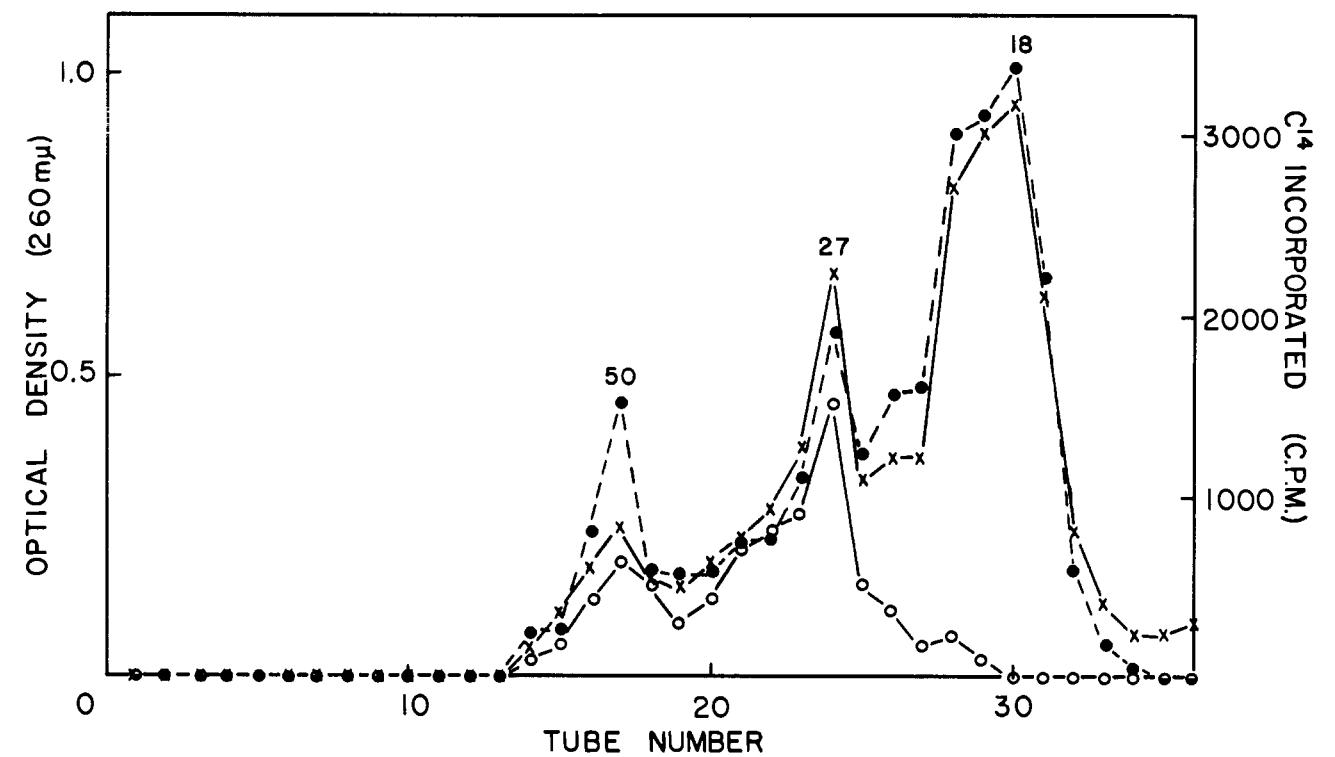

FIG. 8.—Centrifugation of [^{14}C]leucine-labeled ribosomes on a sucrose gradient containing 5×10^{-5} M Mg^{2+}. The symbols are those of Fig. 5.

remained at low Mg^{2+} concentrations, and that protein synthesis was virtually abolished under these conditions. In this connection it was pertinent to assay the activity of the individual ribosomal subunits. In Figure 10, ribosomes fractionated in 5×10^{-5} M Mg^{2+} were tested in the standard incubation mixture containing 5×10^{-3} M Mg^{2+}. Under these conditions the small residual quantity of 50 S ribosomes promoted the formation of both peptide and protein. Neither the 27 nor the 18 S particle was active alone in protein synthesis. However, the 18 S subunit supported peptide formation. The activity of this 18 S fraction was quite comparable to that of the 32 S ribosome separated in 5×10^{-3} M Mg^{2+} and to the peptide-synthesizing ability remaining in 5×10^{-5} M Mg^{2+} in the dialyzed preparations. The small amount of activity under the 27 S peak was prob-

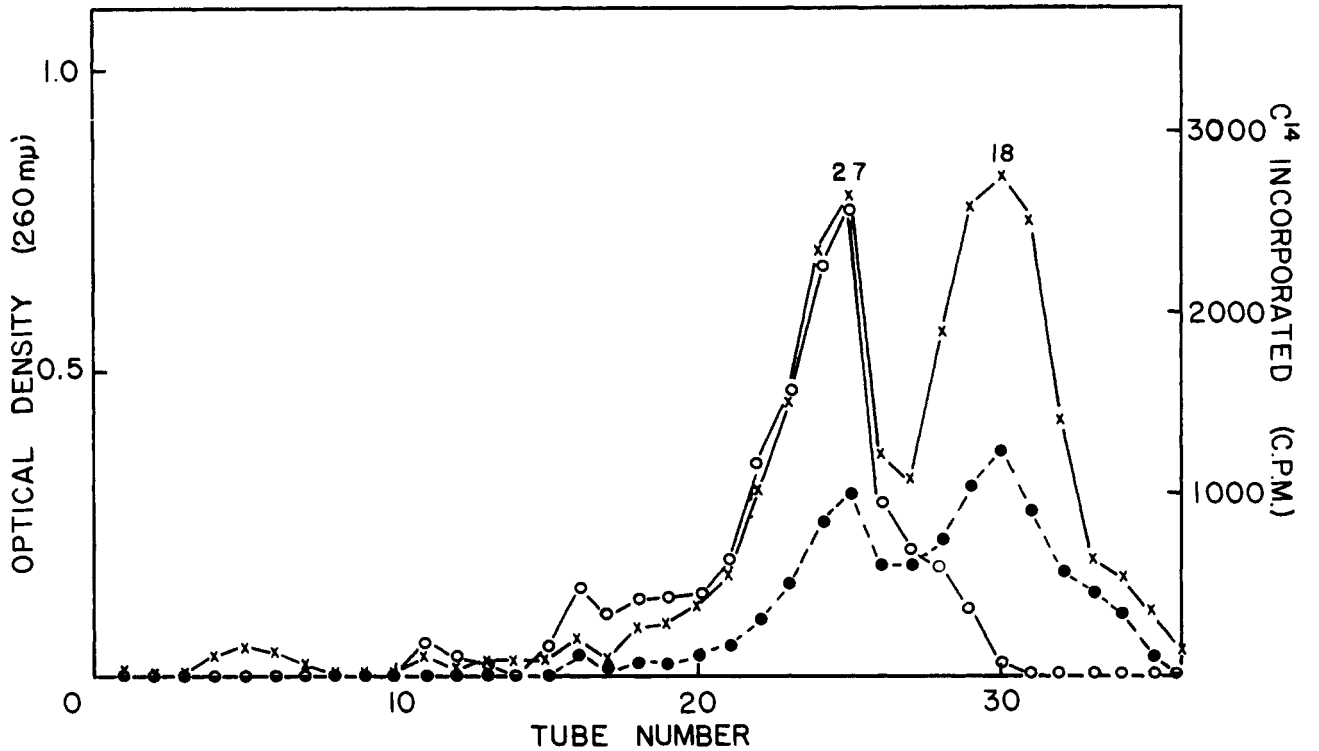

FIG. 9.—Centrifugation of [C^{14}]alanine-labeled ribosomes on a sucrose gradient containing 5×10^{-5} M Mg^{2+}. The symbols are those of Fig. 5.

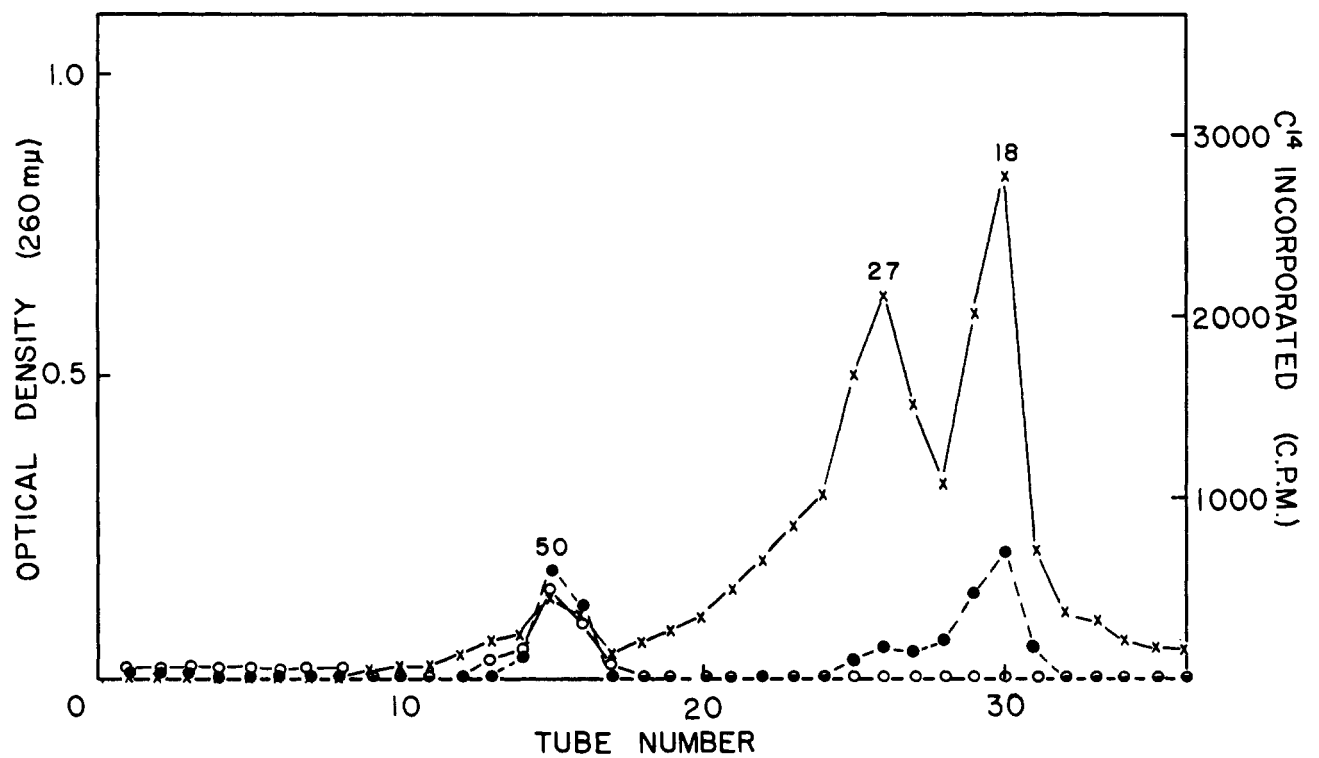

FIG. 10.—Assay of biosynthetic activity of individual ribosomal fractions, following sucrose-gradient fractionation in the presence of 5×10^{-5} M Mg^{2+}. The conditions and symbols are those of Fig. 6, except that DL-[1-^{14}C]leucine was used.

ably owing to undissociated 32 S ribosomes, which would appear at almost the same place. These results have been confirmed using [^{14}C]alanine, so there appears to be little doubt as to their validity. Additionally, it was found that a recombination of aliquots from the 27 and 18 S peaks in 5×10^{-3} M $MgCl_2$ resulted in the restoration of peptide and protein biosynthetic activity to the original level, owing, presumably, to the reconstitution of the 50 S particle.

We wished to determine the stability and reproducibility of the particles (nominally referred to as 18 and 27 S) which were separated on low Mg^{2+}-sucrose gradients, since these particles were relatively unstable when prepared in simple buffer solution by the dialysis

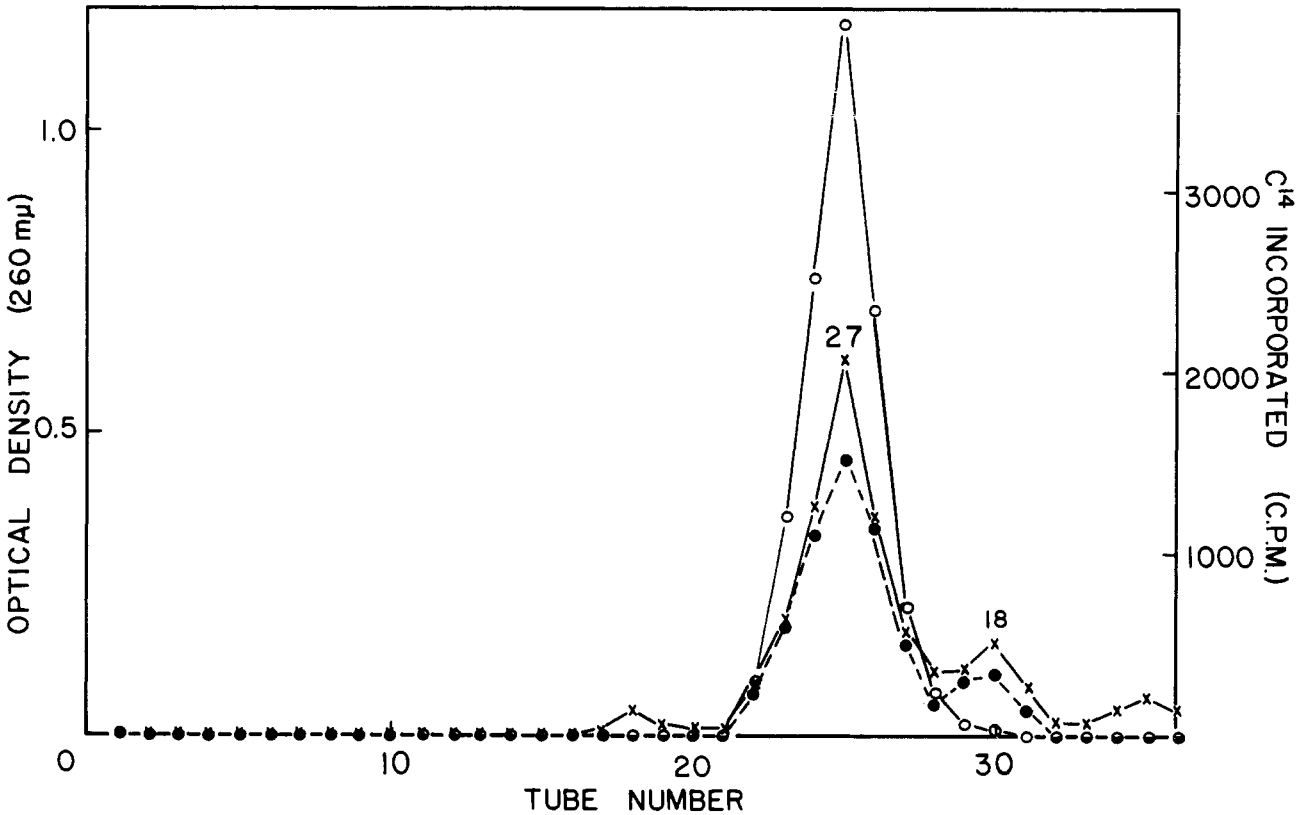

FIG. 11.—Recentrifugation of the [^{14}C]leucine-labeled 27 S ribosomal subunit. The symbols are those of Fig. 5.

technique. For this purpose the individual fractions of the 27 S peak (obtained as in Fig. 8) were combined, and again subjected to the sucrose-gradient procedure at 5×10^{-5} M Mg^{2+} concentration. A very sharp peak resulted (Fig. 11) in the same position as that observed with the original gradient. This peak contained both radioactive peptide and protein. Only a very minor proportion of the 18 S component was present.

Similar studies were performed on the 18 S subunit. In this case, unlabeled ribosomes were separated on a low Mg^{2+}-sucrose gradient, and the 18 S fractions combined. Half the resulting material was then refractionated on a gradient containing 5×10^{-5} M $MgCl_2$, while the other half was centrifuged on a gradient containing 5×10^{-3} M Mg^{2+}. The biosynthetic activity of the fractions from both gradients was then assayed at 5×10^{-3} M Mg^{2+} concentration. The results of this experiment are given in Figure 12.

Following recentrifugation at the low Mg level (Fig. 12a), only a small amount of 27 S component was detected, and this material did not promote [^{14}C]amino acid incorporation into peptide. However, the large 18 S peak had peptide-synthesizing activity. None of the individual fractions supported [^{14}C]leucine incorporation into protein.

At high Mg^{2+} concentration (Fig. 12b), the 18 S peak was slightly reduced in size, but retained peptide-synthesizing activity. The 27 S peak was absent, while a small quantity of the 50 S ribosomal component appeared. In agreement with previous results this larger particle was active in both peptide and protein synthesis. The 32 S ribosome was not reconstituted.

DISCUSSION

In addition to substantiating the obligatory requirement for ribosomes in the cell-free biosynthesis of the gramicidins and tyrocidines, the present work suggests an acceptor role for a particulate material sedimenting in 30 minutes at 40,000 × *g*. This low-speed fraction was itself inactive in either protein or peptide synthesis, but when combined with ribosomes in the presence of a soluble factor(s) it appeared to accept peptides, but not proteins, from their site of synthesis on the ribosomes. This transfer process was not further stimulated by the addition of an energy source, and the required soluble factor(s) was at least partially heat labile. The 40,000 × *g* pellet was obviously heterogeneous, in that it did not yield a sedimentation pattern in the ultracentrifuge. This coarse fraction was clearly distinct from ribosomes on the basis of chemical composition and biological activity. In addition, the 40,000 × *g* pellet contained a considerable concentration of polypeptides. This finding is reminiscent of the report by Chern (1960) that gramicidin J accumulated on the "ghosts" of the Nagano strain of *B. brevis*. The foregoing observations suggest the possibility that cell-membrane fragments were a major component of the low-speed fraction. Since polypeptide production proceeded well in the absence of the 40,000 × *g* material, the latter was omitted in assays of ribosomal activities.

When it became evident from sucrose-gradient experiments that *B. brevis* ribosomes differed in certain aspects from those reported for other systems, such as *E. coli* (Gilbert, 1963), sedimentation analyses were performed to clarify the nature of the particle observed. With crude sonic extracts of *B. brevis*, a major component sedimenting at 50 S was observed. In addition, a small amount of a 32 S particle was present, as well as minor amounts of faster components in some preparations. The use of alumina grinding with lower Tris and higher Mg concentrations (Tissières *et al.*, 1959) led to the detection of increased proportions of particles sedimenting in the range 70–100 S. However, these particles did not appear to be as stable as the 50 S ribosome. The latter was unchanged by storage in the cold for at least 3 weeks, or by 4-hour incubation at 37° in 0.1 M Tris and 5×10^{-3} M Mg^{2+}. In control

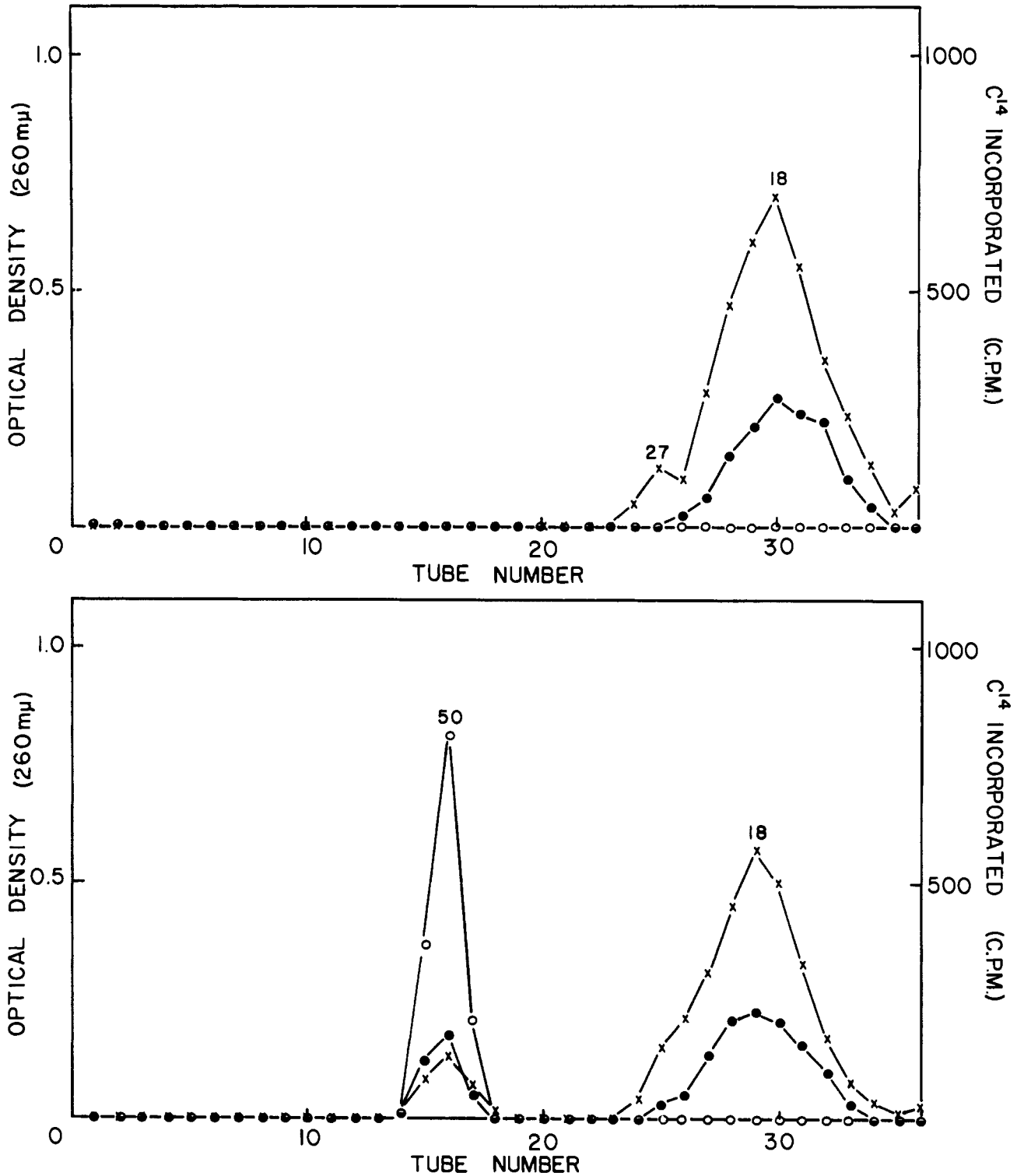

FIG. 12.—Assay of 18 S subribosomes following recentrifugation on sucrose gradients. Gradients contained: (a) (upper) 5×10^{-6} M Mg^{2+}; and (b) (lower) 5×10^{-3} M Mg^{2+}. DL-[1-^{14}C]leucine was employed in the assays under standard conditions. The symbols are those of Fig. 5.

experiments, crude extracts of *E. coli* produced the usual pattern of 100, 70, 50, and 30 S particles (Tissières *et al.*, 1959; Huxley and Zubay, 1960). Unlike the *B. brevis* ribosomes with similar sedimentation coefficients, the 50 and 30 S *E. coli* subunits occurred always in a ratio of 2:1. The dissociation and reaggregation of the *E. coli* particles were freely reversible, while *B. brevis* ribosomes remained predominantly in the 50 S form at Mg^{2+} concentrations well in excess of the optimum for biosynthetic activity.

When *B. brevis* preparations were dialyzed against low Mg^{2+} concentrations in the presence of 0.01 M Tris, a new particle was observed with a sedimentation value of about 40–45 S, which apparently originated at the expense of the 50 S ribosome. This observation may be analogous to that of Elson (1961) with *E. coli* preparations, but we were unable to demonstrate the release of a 4–5 S component with the crude extracts employed.

Dialysis against low Mg^{2+} concentrations in 0.1 M Tris buffer produced markedly different results. At least two subunits were observed. The particles, nominally referred to as 18 and 27 S, were apparently derived

from the 50 S ribosome, since the latter accounted for about 90% of the ribosomal material in the undialyzed preparations. The significance of these small components has been difficult to assess, since their sedimentation coefficients decreased with increasing times of dialysis, and the high buffer concentrations apparently activated a latent ribonuclease. The gradual decrease in S values suggested possible conformational changes. Despite the anomalies arising in buffer solution, reproducible data were obtained for the analogous particles obtained on sucrose gradients.

When *B. brevis* preparations were sedimented on sucrose gradients containing 0.1 M Tris and 5×10^{-3} M Mg^{2+}, only 50 and 32 S particles were observed, even though in some cases the same ribosomal preparations exhibited faster particles on the analytical centrifuge in the absence of sucrose. Gilbert (1963) has shown that either the 70 S ribosome or a combination of 50 and 30 S subunits was required for isotopic amino acid incorporation into protein, with an *E. coli* system. In the case of *B. brevis* ribosomes, the 50 S particle was active in peptide and in protein formation. Furthermore, the 32 S particle also had a significant amount of peptide-forming ability. Although we have not tested a recombination of 32 and 50 S particles, the amount of synthesis by the individual components corresponded approximately to that observed with unfractionated systems.

B. brevis ribosomes, separated on gradients containing high (0.1 M) Tris and low (5×10^{-5} M) Mg^{2+} concentrations, yielded a consistent pattern: 18 and 27 S peaks. When labeled ribosomes were fractionated under these conditions, [^{14}C]protein was associated exclusively with the 27 S unit, while [^{14}C]peptide occurred in both peaks. An examination of the biosynthetic activity of these particles revealed that neither component was active alone in protein formation. However, a recombination of the two subunits led to a complete restoration of protein, as well as peptide, biosynthesis. A reformation of the 50 S ribosome apparently occurred. The 18 S particle showed approximately the same total peptide-synthesizing activity as did the 32 S ribosome. This correspondence suggested a relationship between these two structures. It is possible that some of the 185 particles were derived from the 32 S ribosomes, either by dissociation or by a conformational change. We have not been able to obtain evidence for the re-formation of the 32 S ribosome.

The data presented in this paper raise a number of interesting questions. Although an active 50 S ribosome was the predominent form in extracts of the Dubos strain of *B. brevis*, the existence of other ribosomal species (in minor amounts) with sedimentation properties similar to the 30, 70, and 100 S particles of *E. coli* seems more than coincidental. In fact, experiments with another strain of *B. brevis* (ATCC 9999) provide strong evidence for the existence in this bacterial species of a native 70 S ribosome similar to that found in *E. coli*, and in other bacterial and mammalian systems (Petermann and Hamilton, 1961). It appears unlikely that the *B. brevis* organism is atypical in this respect, but our present data do not entirely exclude such a possibility. However, the observation (J. D. Watson, personal communication) that the 30 S particle from some bacterial species is quite susceptible to proteolytic digestion might offer an explanation for our inability to detect large amounts of the 30 as well as the 70 S ribosome.

The physical significance of the 18 and 27 S particles arising from the 50 S ribosome is not presently clear. It is possible that these structures may bear some relationship to the small *E. coli* subunits observed by other investigators (Bowen *et al.*, 1959; Rodgers, 1964).

Finally, it is hoped that future studies may throw light upon the intriguing problem of the basic physicochemical structure of ribosomes concerned with biosynthesis of natural polypeptides, and on the mode of participation of these units in the intimate mechanism of peptide-bond formation.

References

Bowen, T. J., Dagley, S., and Sykes, J. (1959), *Biochem. J. 72*, 419.

Britten, R. J., and Roberts, R. B. (1960), *Science 131*, 32.

Chern, C. J. (1960), *J. Osaka City Med. Center 9*, 2861.

Elson, D. (1961), *Biochim. Biophys. Acta 53*, 232.

Gilbert, W. (1963), *J. Mol. Biol. 6*, 148.

Huxley, H. E., and Zubay, G. (1960), *J. Mol. Biol. 2*, 10.

Leonard, C. G., and Housewright, R. D. (1963), *Biochim. Biophys. Acta 73*, 531.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Okuda, K., Edwards, G. C., and Winnick, T. (1963), *J. Bacteriol. 85*, 329.

Okuda, K., Saito, Y., and Otani, S. (1960), *J. Japan. Biochem. Soc. (Seikagaku) 32*, 650.

Okuda, K., Uemura, I., Bodley, J. W., and Winnick, T. (1964a), *Biochemistry 3*, 108.

Okuda, K., Uemura, I., Bodley, J. W., and Winnick, T. (1964b), *Biochemistry 3*, 100.

Petermann, M. L., and Hamilton, M. G. (1961), *Protein Biosyn. Symp. Wassernaar, Neth., 1960*, 233.

Rodgers, A. (1964), *Biochemistry J. 90*, 548.

Schneider, W. C. (1957), *Methods Enzymol. 3*, 680.

Strominger, J. L. (1962), *Federation Proc. 21*, 134.

Tissières, A., Watson, J. D., Schlessinger, D., and Hollingworth, B. R. (1959), *J. Mol. Biol. 1*, 221.

Uemura, I., Okuda, K., and Winnick, T. (1963), *Biochemistry 2*, 719.