CHARACTERIZATION OF RIBOSOMES FROM *NEUROSPORA CRASSA*

R. STORCK

From the Department of Microbiology, The University of Texas, Austin

ABSTRACT Ribosomes isolated from growing hyphae of *Neurospora crassa* contain 53 per cent protein and 47 per cent RNA and have a sedimentation coefficient of 81S at 20°C and infinite dilution. These ribosomes are stable at pH 7.4 in the presence of 0.01 M and 0.002 M $MgCl_2$ but undergo a dissociation into smaller particles if the $MgCl_2$ concentration is lowered to 0.0001 M. Two types of RNA with sedimentation coefficients of $19S_{20}^{20}$ and $13S_{20}^{20}$ have been extracted from the 81S particles.

A. INTRODUCTION

Ribosomes are ribonucleoprotein particles (RNP) having sedimentation coefficients ranging from 30S to 150S. These RNP tend to associate in the presence of a high concentration of dibasic metal ions. Dissociation occurs when this concentration is lowered. In the case of Mg ions, dissociation usually takes place at concentrations ranging between 0.001 M and 0.0001 M (1). In intact cells, the ribosome population can be changed by modifying the physiological stage (2-5). Recent experiments performed with *in vivo* (6, 7) and *in vitro* systems (8-10) strongly suggest that ribosomes are the primary site of protein synthesis.

RNP have been found in extracts of all the animal, plant, and microbial cells so far analyzed (1, 11). In many instances careful studies of the physicochemical properties of these ribosomes have been made. In fungi, however, no such information is yet available with the exception of yeast (12) and *Penicillium funiculosum* (13). The presence of RNP in hyphae of *Neurospora crassa* has been demonstrated by electron microscopy (14, 15). Several authors have obtained ribosomes in pellets during differential centrifugation of mycelial and conidial extracts (16-18).

In the present work, ribosomes were isolated from growing hyphae of *N. crassa* and identified as RNP with a sedimentation coefficient of 81S. These particles, stable at pH 7.4 in 0.002 M Mg^{++}, dissociate into two smaller components when the Mg^{++} concentration is lowered to 0.0001 M. The 81S particles contain two RNA types with sedimentation coefficients of 19S and 13S. Thus *N. crassa* ribosomes have the general characteristics of RNP found in other organisms and are similar to those which have been isolated from yeast.

B. METHODS

1. *Growth of N. crassa.* The strain used in this work was kindly supplied by Dr. R. P. Wagner. It is a wild type obtained in his laboratory from a cross between wild types 5256 A and 5297 a. Both strains originate from the laboratory of Dr. S. Emerson of the California Institute of Technology.

N. crassa was grown on Vogel's synthetic medium supplemented with 2 per cent sucrose (19). Conidia from 7-day-old cultures on solid medium were collected aseptically with sterile medium. The spore suspension was then homogenized to destroy conidia aggregates and stored in the freezer. The number of conidia per milliliter was routinely determined by measuring the turbidity of the suspension with a Klett-Summerson colorimeter using a No. 56 filter. The measurements were made after determining the proportionality zone of the curve obtained by plotting turbidity *versus* the reciprocal of several dilutions of a spore suspension. The number of conidia per milliliter was determined directly with a bacteria counter. The dry weight of spores washed twice with distilled water was also determined. Standard curves were then obtained by plotting turbidity values *versus* spore number and dry weight. It was found that 1 colorimetric unit corresponds to 8×10^4 spores/ml and 4.8×10^{-3} mg dry weight/ml. The average dry weight of one conidium is thus equal to 6×10^{-5} μg, 57 per cent of which is protein and 2 per cent phosphorus.

A liquid medium was inoculated with spore suspensions of a known turbidity. The cultures were incubated at 30°C on a rotary shaker. To obtain maximum aeration and prevent the formation of large mycelial aggregates, long-necked Erlenmeyer flasks with baffles were used. At the end of a 10 to 13 hour lag phase the spores germinated, each giving rise to an individual mycelium. After given time intervals samples were removed and their turbidity recorded. A typical growth curve is shown in Fig. 1. The initial

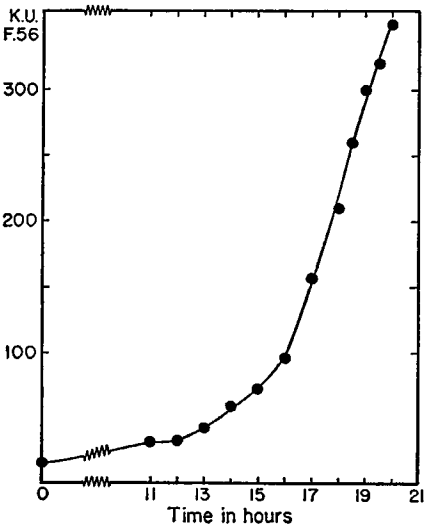

FIGURE 1 Growth curve of *Neurospora crassa*. 400 ml of medium was inoculated with 10 ml of a spore suspension having a turbidity corresponding to 640 Klett units (Filter No. 56). Samples were removed at the times indicated and their turbidity recorded. When the turbidity reading was higher than 100 Klett units, the sample was diluted in order to remain in the linear portion of the calibration curve (Klett units *versus* dilution).

turbidity rise corresponds to the swelling of the conidia and the formation of a germination tube followed by an increase in the volume of the mycelium originating from each spore. A rapid increase in turbidity typifies the second phase of growth. A straight line

is obtained if the cubic root of the turbidity values obtained in this last growth phase are plotted *versus* time. The same type of curve is obtained when the cubic root of the dry weight is plotted *versus* time (20).

Since the method used in the present work for growing *N. crassa* is the same as that used for growing bacteria and yeast, an accurate sampling is possible. This method is now being used for the study of the kinetics of RNA synthesis.

2. *Preparation of Mycelial Extracts.* Mycelia from cultures having a turbidity corresponding to about 3 mg/ml were centrifuged and washed twice with 0.01 M tris(hydroxymethyl)-amino methane HCl buffer at pH 7.4 containing 50 μmoles of KCl/ml (tris-KCl). The pellets were pressed between sheets of filter paper to remove excess water. The hyphae were disrupted at 0°C in the presence of 0.01 M $MgCl_2$ either by alumina grinding or by shaking with glass beads in a Nossal disintegrator. The abrasives and large cell debris were removed by centrifuging twice at 15,000 *g* for 15 minutes. The supernatant (15 Gs) containing tris-HCl buffer at pH 7.4 and $MgCl_2$ both at a concentration of 0.01 M (tris-Mg) was used for the isolation of ribosomes. The concentration of the 15 Gs is expressed in RNA assuming that an optical density at 260 mμ (OD_{260}) of 1.0 corresponds to 50 μg.

3. *Detection, Isolation, and Characterization of Ribosomes.* The 15 Gs was centrifuged in a linear gradient of sucrose in tris-Mg (21). Fractions of equal volume were collected after centrifugation and their OD_{260} measured. A profile was obtained by plotting OD_{260} *versus* fraction number. The fractions corresponding to the upper part of the ribosomal peaks were pooled. Ribosomes were pelleted and washed three times with tris-Mg by centrifugation at 40,000 RPM for 180 minutes in the rotor No. 40 of the Spinco model L preparative ultracentrifuge. The determinations of the sedimentation coefficients were performed with a Spinco model E analytical ultracentrifuge equipped with Schlieren optics.

4. *Preparation and Characterization of RNA.* RNA was obtained from ribosomes after extraction with sodium dodecyl sulfate and protein elimination by phenol (23). RNA in potassium acetate (0.2 M) was further purified by repeated ethanol precipitation. The sedimentation analysis of ribosomal RNA was performed with the ultraviolet adsorption system of the Spinco model E ultracentrifuge. The sedimentation coefficients were normalized to a temperature of 20°C. The concentration of RNA solutions was measured by determination of the OD_{260} using an equivalence of 50 μg/unit OD_{260}.

5. *Chemical Analysis.* Protein was determined by the method of Lowry *et al* (24) and RNA by the method of Mejbaum (25). Crystalline lysozyme and bovine serum albumin were used as standards for protein determination. Ribose was used as a standard for the determination of RNA.

6. *Preparation of Labeled Ribosomes and RNA from Escherichia coli.* For comparative purposes radioactive ribosomes and RNA were used as markers in the density gradient centrifugation of *N. crassa* extracts. An exponentially growing culture of *E. coli* was allowed to incorporate H^3-uridine for 10 minutes. The culture was harvested by centrifugation and washed twice with tris-KCl containing 0.01 M sodium azide (22). The cells were disrupted by grinding with alumina in the presence of 0.01 M $MgCl_2$ and 10 μg DNase/ml. Unbroken cells and cell wall debris were removed by two centrifugations at 15,000 *g* for 15 minutes. The clear supernatant (15 Gs) was centrifuged in a sucrose density gradient. Fractions were collected and their OD_{260} measured. Aliquots were removed, mixed with 250 μg of DNA, and precipitated with trichloroacetic acid. The precipitates were collected on filter membranes, washed, dried, and the

radioactivity measured in a liquid scintillation spectrometer (27). In the centrifugation diagrams OD_{260} and radioactivity peaks corresponding to ribosomes coincided. The specific activity of these peaks had an average value of 500 CPM/μg RNA.

C. RESULTS

1. *Characterization of N. crassa Extracts by Centrifugation in a Sucrose Density Gradient.* *N. crassa* and *E. coli* 15 Gs containing respectively 1 mg and 90 μg of RNA were mixed and layered on a 3 per cent to 20 per cent sucrose gradient. After 120 minutes' centrifugation at 38,000 RPM the OD_{260} and the radioactivity of the collected fractions were determined. Two profiles were obtained and they are shown in Fig. 2. The radioactivity profile produced by *E. coli* is char-

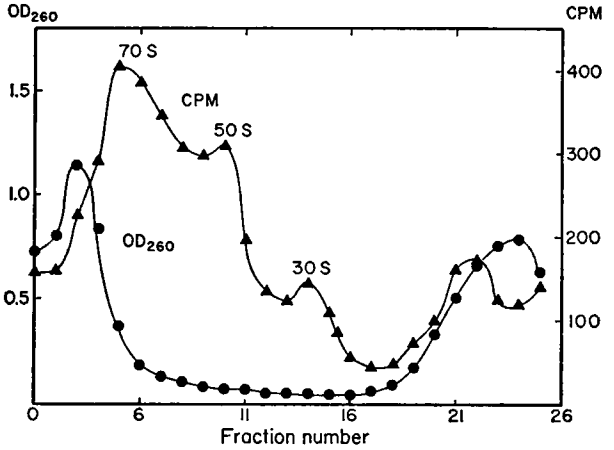

FIGURE 2 A mixture of *N. crassa* and *E. coli* extracts containing respectively 1 mg and 90 μg of RNA were layered on top of a 3 per cent to 20 per cent linear sucrose gradient. The mixture was centrifuged for 120 minutes at 38,000 RPM. The two profiles were obtained after collecting fractions of equal volume and measuring both their OD_{260} and their radioactivity. The RNA of *E. coli* was labeled with H^3-uridine. The OD_{260} profile corresponds to the *N. crassa* extract.

acterized by three ribosomal peaks with respective sedimentation coefficients of 70S, 50S, and 30S. A fourth peak produced by soluble RNA and proteins is also present in the profile. The OD_{260} profile produced only by *N. crassa* is characterized by two peaks. The peak situated at the top of the profile corresponds to soluble RNA and proteins. The peak located near the bottom of the centrifuge tube is produced by *N. crassa* ribosomes. They are larger in size than the 70S *E. coli* ribosomes. Little or no ultraviolet adsorption occurs between the two OD_{260} peaks. Such a centrifugation diagram could result from a technical artifact rather than the typical property of *N. crassa*. The influence of several factors on the centrifugation profile was analyzed to validate this assumption.

The centrifugation profile was not modified when extracts were prepared from cultures with turbidities ranging from 150 to 600 Klett units nor when the centrifugation time was changed from 80 minutes to 120 minutes nor when the RNA concentration of the 15 Gs layered on top of the sucrose gradient varied from 0.5 to 1.5 mg/ml. The OD_{260} profile was further found independent of the method used for the layering of the 15 Gs on top of the sucrose gradient: by direct layering, in a mixture with 2 per cent sucrose; or by inverted gradient (26). Furthermore, the method used for destruction of the hyphae was found to have no effect on the centrifugation diagram. However, since large amounts of alumina and glass beads (5 gm and 40 gm per gram dry weight of mycelium, respectively) were needed to obtain a good extraction yield, some ribosomes could possibly have been adsorbed on these abrasives. The centrifugation pellets containing alumina or glass beads were therefore washed several times by centrifugation in tris-Mg. After these washings, pellets and supernatants were analyzed for their RNA and protein content.

The results expressed as per cent of the sum of all the fractions analyzed show that for a 15 Gs corresponding to 67 per cent RNA and protein the first washing represented 22.5 per cent to 24 per cent, the second 3.0 per cent to 4.5 per cent, and the third 3.0 per cent to 4.0 per cent. The remainder, 2.0 per cent to 3.0 per cent, were accounted for by the pellet. When the first washing was centrifuged through a sucrose density gradient, it yielded a profile identical with that obtained with the 15 Gs. There remained the possibility that in *N. crassa* some ribosomes are firmly attached to an endoplasmic reticulum (16). Therefore, after grinding the mycelium, the pellets containing alumina or glass beads and large cell debris were treated with 0.5 per cent sodium deoxycholate or two volumes of trifluorotrichloroethane. After centrifugation through a sucrose density gradient under conditions similar to those described above, a profile identical to that shown in Fig. 2 was obtained. Therefore the small amounts of protein and RNA found in the centrifugation pellets do not correspond to ribosomes of a class different from that observed in the diagram of the density gradient centrifugation.

As already indicated, ribosomes can be characterized by the dissociation they undergo when the Mg ion concentration is decreased to a specific critical value. *N. crassa* extracts in tris-Mg were therefore dialyzed against 0.01 M, 0.002 M and 0.0001 M Mg^{++} for a period of 16 hours. As shown in Fig. 3, the OD_{260} profile obtained after density gradient centrifugation is not affected by dialysis against 0.01 M Mg^{++}. The same observation was made when the Mg^{++} concentration was 0.002 M. However, when extracts originally in 0.01 M were dialyzed for the same period of time against 0.0001 M Mg^{++}, the single ribosome peak disappeared and was replaced in the density gradient centrifugation profile by two distinct major peaks corresponding to particles of smaller size. After 15 hours' dialysis of such an extract against 0.01 M Mg^{++}, an incomplete reassociation of the two smaller components leading to the reformation of the larger RNP peak was observed.

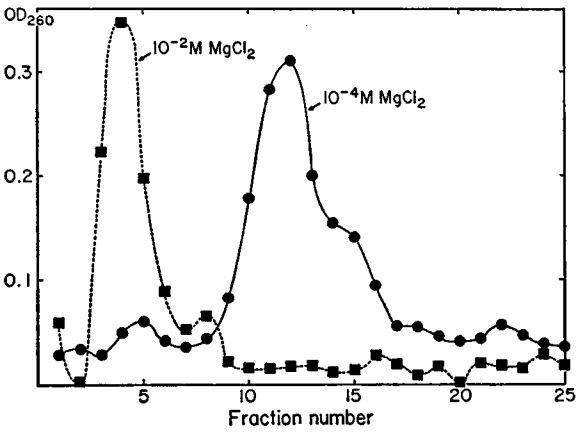

FIGURE 3 Two preparations of *N. crassa* ribosomes having identical OD_{260} values and containing tris-Mg (0.01 M $MgCl_2$) were dialyzed for 16 hours, one against 0.01 M $MgCl_2$ (dotted line) and the other against 0.0001 M $MgCl_2$ (solid line). After dialysis, the preparations were centrifuged at 38,000 RPM for 100 minutes in a 3 per cent to 20 per cent linear sucrose gradient with the appropriate Mg^{++} concentration. The preparation dialyzed against 0.01 M $MgCl_2$ gives a normal profile (see Fig. 2). After dialysis against 0.0001 M $MgCl_2$, the large ribosomes disappeared and gave rise to two new peaks corresponding to particles with a smaller sedimentation coefficient.

2. *Determination of Sedimentation Coefficients.* Four different concentrations of 15 Gs, ranging from 625 μg to 5,000 μg RNA/ml were centrifuged at 39,460 RPM and 20°C in the analytical ultracentrifuge. With 625, 1,250, and 2,500 μg RNA/ml two peaks were observed as in the density gradient centrifugation profiles. As shown in Fig. 4, a value of 81.2 is obtained when the curve obtained by plotting S values against concentrations is extrapolated to zero concentration.

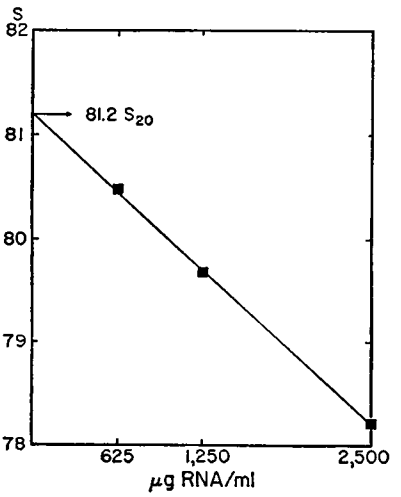

FIGURE 4 Determination of the sedimentation coefficient of *N. crassa* ribosomes. Three preparations containing respectively 625, 1,250, and 2,500 μg RNA/ml were centrifuged at 39,460 RPM and 20°C. The individual values obtained for the sedimentation coefficient are plotted *versus* concentration. The extrapolation of the curve to zero concentration corresponds to a value of $81.2S_{20}$.

By increasing the concentration of RNA to 5,000 μg/ml, two small additional peaks could be detected. These peaks were located between the 81S component peak and the soluble material peak. A determination of the sedimentation coefficient without correction for concentration gave respective values of 39S and 49S. These values are too low because of the interference caused by the very high concentration of 81S ribosomes. A comparison of the area under the curves indicated that these two small particle types represent less than 1 per cent of the total ribosome population.

Purified RNA extracted from 81S RNP was centrifuged at 59,730 RPM and 26°C. As can be seen in Fig. 5, two boundaries are present in the microdensitometer

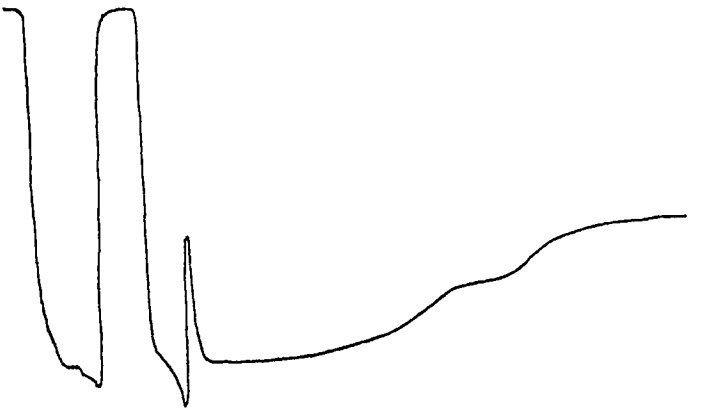

FIGURE 5 RNA extracted from *N. crassa* ribosomes by the sodium dodecyl sulfate phenol method and purified by repeated ethanol precipitation was dissolved in tris-KCl. The preparation containing 28 μg RNA/ml was centrifuged at 59,730 RPM and 26°C in the analytical ultracentrifuge equipped with ultraviolet optics. The figure shows a typical microdensitometric tracing with two boundaries corresponding to 19S and 13S RNA, respectively.

tracing. These boundaries correspond respectively to $19.09S_{20}^{50}$ and $12.76S_{20}^{50}$ RNA classes.

3. *Chemical Analysis.* Ribosome pellets washed three times with tris-Mg were analyzed for their relative RNA and protein content. An average ratio of OD_{260} over OD_{280} of 1.86 was found. Repeated chemical determinations on samples originating from several extracts yielded an average of 53.4 per cent protein and 46.6 per cent RNA.

D. DISCUSSION

Ribosomes have been found in cells of animals, plants, and bacteria. These ribonucleoprotein particles display a remarkable uniformity in their properties. They contain RNA and protein in almost equal proportions. They have similar molecular

weights and their average sedimentation coefficient lies around 80S. At neutral pH when the Mg^{++} concentration is lowered to 0.0001 M they undergo a dissociation into smaller particles characterized by sedimentation coefficients of 40S and 60S.

The results presented in this work show that *N. crassa* is not an exception to the list of the organisms studied so far.

The only fungus in which ribosomes have been studied in detail is yeast (12, 28, 29, 30). Therefore, a comparison of the properties of the RNP from yeast (primarily the genus *Saccharomyces*) and *N. crassa* might be more instructive. The ribosomes of yeast have a sedimentation coefficient of 80S and those of *N. crassa* 81S. The ratio of OD_{260} over OD_{280} is the same for both types of particles. In both organisms the RNA content of the ribosomes is about 40 per cent. Certainly the results of the chemical analysis presented in this work are not absolutely correct since washing with buffer does not necessarily remove aggregated protein. However, the 81S particles described here are obviously of a ribonucleoprotein nature and can therefore be referred to as ribosomes. *N. crassa* ribosomes could thus have a molecular weight like that of yeast between 4.1×10^6 and 4.5×10^6 (12). The control of the reversible dissociation of these particles is achieved by similar Mg ion concentrations. The sedimentation coefficient of the dissociation products of the 81S particles was not determined directly. However, a comparison between the position of the derived *N. crassa* ribosomes and the derived 30S and 50S of *E. coli* indicates that they have sedimentation coefficients corresponding to 60S and 40S, respectively. These values are in agreement with those found for the dissociation products of the 80S yeast ribosomes (12). Purified ribosomal RNA from yeast falls into two categories: 28S and 19S (30). In *N. crassa* the principal RNA class was found to have sedimentation coefficient of 19S. This value is not unique for yeast and *N. crassa* since it is also characteristic of ribosomal RNA extracted from plant and animal cells (1). Recently experimental evidence has been presented (30) which indicates that the fundamental unit of yeast ribosomal RNA has a sedimentation coefficient of 19S and that 28S molecules are dimers formed in the presence of Mg^{++} concentration on the order of 0.01 M. It has also been shown (1) that in the absence of Mg^{++} ions, 19S RNA undergoes a dissociation into smaller molecules, some of which have a sedimentation coefficient of 13S. The absence of 28S molecules and the presence of 13S RNA in our preparations could thus be explained by use of tris-KCl buffer as a solvent. Certainly the comparison between the position of *E. coli* and *N. crassa* ribosomal RNA in gradient centrifugation profiles (when large amount of RNA is used) indicates the presence of a molecular class which sediments faster than the 23S RNA characteristic for *E. coli*.

In higher organisms, ribonucleoprotein particles are attached to an endoplasmic reticulum. They are obtained as free particles after treatment of the cells with deoxycholate. Generally when such a treatment is unnecessary as for embryonic,

tumor, and bacterial cells, the absence of an internal membrane organization is indicated. Our results suggest that the same situation prevails for hyphae of *N. crassa.* However, fine structure studies with the electron microscope (14, 15) demonstrate an internal membrane similar to the endoplasmic reticulum of higher organisms. Possibly this internal structure is absent only from young growing hyphae of *N. crassa.* Such a conclusion is in part supported by the fact that ribosomes are extractable from *N. crassa* conidia (31) and *Aspergillus niger* hyphae (32) only after deoxycholate treatment of the disrupted cells. Fine structure studies with the electron microscope will be undertaken to verify this observation directly.

We are indebted to Dr. S. Spiegelman and Dr. C. Doudney for placing their analytical ultracentrifuge at our disposal.

This work was supported by the United States Public Health Service and The University of Texas Research Institute.

Received for publication, May 21, 1962.

REFERENCES

1. PETERMAN, M. L., and HAMILTON, M. G., in Protein Biosynthesis (R. J. C. Harris, editor). New York, Academic Press, Inc., 1961, 233.
2. MCCARTHY, B. J., *Biochim. et Biophysica Acta,* 1959, **39,** 573.
3. WOESE, C. R., *J. Bact.,* 1961, **82,** 695.
4. PLESNER, P., *Cold Spring Harbor Symp. Quant. Biol.,* 1961, **26,** 159.
5. LOENING, U. E., *Biochem. J.,* 1961, **81,** 254.
6. MCQUILLEN, K., Roberts, R. B., and BRITTEN, R. J., *Proc. Nat. Acad. Sc.,* 1959, **45,** 1437.
7. BRENNER, S., *Cold Spring Harbor Symp. Quant. Biol.,* 1961, **26,** 101.
8. TISSIERES, A., SCHLESSINGER, D., and GROS, F., *Proc. Nat. Acad. Sc.,* 1960, **46,** 1450.
9. WEBSTER, B., and LINGREL, J. B., *in* Protein Biosynthesis (R. J. C. Harris, editor), New York, Academic Press, Inc., 1961, 301.
10. NIRENBERG, M. W., and MATTHAEI, J. H., *Proc. Nat. Acad. Sc.,* 1961, **47,** 1588.
11. ROBERTS, R. B., editor, Microsomal Particles and Protein Synthesis, New York, Pergamon Press, Inc., 1958.
12. CHAO, F. C., and SCHACHMAN, H. K., *Arch. Biochem. and Biophysics,* 1956, **61,** 220.
13. LEWIS, V. J., RICKES, E. L., MCCLELLAND, L., and BRUIK, N. B., *J. Am. Chem. Soc.,* 1959, **81,** 4115.
14. ZALOKAR, M., *J. Biophysic. and Biochem. Cytol.,* 1961, **9,** 609.
15. SCHATKIN, A. J., and TATUM, E. L., *J. Biophysic. and Biochem. Cytol.,* 1959, **6,** 423.
16. SCHULMAN, H. M., and BONNER, D. M., *Proc. Nat. Acad. Sc.,* 1962, **48,** 53.
17. WAINWRIGHT, S. D., *Canad. J. Biochem. and Physiol.,* 1959, **37,** 1417.
18. HAROLD, F. M., and MILLER, A., *Biochim. et Biophysica Acta.,* 1961, **50,** 261.
19. VOGEL, H. J., *Microbial Genetics Bull.,* 1956, 13.
20. EMERSON, S., *J. Bact.,* 1950, **60,** 221.
21. BRITTEN, R. J., and ROBERTS, R. B., *Science,* 1960, **131,** 32.
22. NOMURA, M., HALL, B. D., and SPIEGELMAN, S., *J. Molecular Biol.,* 1960, **2,** 306.
23. KURLAND, C. G., *J. Molecular Biol.,* 1960, **2,** 83.
24. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., and RANDALL, R. J., *J. Biol. Chem.,* 1951, **193,** 265.
25. VOLKIN, E., and COHN, W. E., *in* Methods of Biochemical Analysis (D. Glick, editor), New York, Interscience Publishers, 1954, 287.

26. ROBERTS, R. B., ABELSON, P. H., COWIE, D. B., BOLTON, E. B., and BRITTEN, R. J., *Carnegie Institution of Washington, Year Book*, **58**, 1959.
27. SPIEGELMAN, S., HALL, B. D., and STORCK, R., *Proc. Nat. Acad. Sc.*, 1961, **47**, 1135.
28. KIHARA, H. K., HU, A. S. L., and HALVORSON, H. O., *Proc. Nat. Acad. Sc.*, 1961, **47**, 489.
29. CHAO, F. C., *Arch. Biochem. and Biophysics*, 1957, **70**, 426.
30. MAEDA, A., *J. Biochem.* (Japan), 1961, **50**, 377.
31. MARTINEK, J., and STORCK, R., data to be published.
32. MOYER, R., and STORCK, R., data to be published.