Ribosomes. A Study of Active and Sluggish Preparations*

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ABSTRACT: The amino acid incorporating activity of yeast ribosomes depends upon the point in the log phase of growth at which the yeast is harvested. The mechanism of this alteration in ribosomal activity has been investigated with the possibility in view that it may represent control of protein synthesis at the ribosomal level. A comparison of active (mid log phase) and sluggish (early log phase) ribosomal preparations showed no differences in the following properties: sedimentation constants, content of ribosomal subunits, base ratios of ribosomal ribonucleic acid (RNA), stability either to standing in the cold or to incubation, effect of washing and the activity of ribosomal wash fluid, and extent of saturation with messenger RNA (m-RNA). A small but consistent difference was found in the RNA/protein ratio of the two preparations. When the two ribosomal preparations were compared with respect to their polysome content, it was found to be identical. However, strong evidence was obtained that polysomes isolated from the sluggish ribosomal preparations are themselves more poorly able to carry out amino acid incorporation than are polysomes from active ribosomal preparations. The factors responsible for this difference are not yet known. In addition to these studies on isolated ribosomes, experiments at the cellular level indicate that substantial variation in the composition of the medium occurs during log phase, and that this variation results in profound changes in the metabolic state of the yeast cell as well as in changes in ribosomal activity.

Recent studies (Lucas *et al.*, 1964) on a ribosomal system derived from *Saccharomyces cerevisiae* have shown that, although these studies have been carried out on logarithmically growing cells, the amino acid incorporating activity of the cell-free system is nevertheless not constant throughout the log phase. It was observed that this variation is a property of the ribosomes themselves, that their activity rises to a peak at approximately mid-log phase and then descends sharply thereafter. This phenomenon does not appear to be confined to yeast; such inconstancy also appears to be the case in *Escherichia coli*, as was first intimated by Lamborg and Zamecnik (1960), further observed by Doerfler *et al.* (1962), and confirmed by us (Dietz, 1965). In addition to the variation in the activity of the yeast cell-free system, the number of ribosomes per cell changes during the log phase, roughly paralleling the changes in ribosomal activity, and this was also noticed in *E. coli* (Doerfler *et al.*, 1962).

Our ultimate concern in this problem lies primarily with pinpointing the locus in the ribosomal machinery which is responsible for this change in ribosomal activity. As a first step in this direction, it was necessary to ascertain whether these differences in activity resulted merely from differences in messenger ribonucleic acid (m-RNA) content and consequently in the polysome to monomer ratio of the different ribosomal preparations, or whether the differences are related to factors inherent in the ribosome.

In this communication active and sluggish ribosomal preparations are compared, and evidence is provided that the sluggish preparations are not less stable and are not lacking in m-RNA, and that the polysome content of sluggish and active preparations are identical; rather, the polysomes themselves possess different incorporating activities. Experiments now in progress are designed to elucidate the functional lesion in the sluggish ribosomes.

In addition to these studies on isolated ribosomes, a few preliminary experiments at the cellular level are reported which point to a relationship between the variation in the composition of the medium during log phase and a number of profound alterations which occur in the metabolism of the cell including the changes in ribosomal activity.

The existence of mechanisms for the control of protein synthesis at the ribosomal level has been hypothesized by many, but little direct evidence is available to support such hypotheses. This system is being studied with such possibilities in view.

Experimental Procedure

Materials. All labeled amino acids were obtained from

* From the Department of Biochemistry, Dartmouth Medical School, Hanover, N. H. *Received May 4, 1965; revised August 2, 1965.* This investigation was supported by a grant (RO1 AM 07124-03 BIO) from the U. S. Public Health Service. A preliminary report of these studies has been published (Dietz and Simpson, 1964). This communication is taken from a dissertation submitted to Yale University in partial fulfillment of the requirements for the Ph.D. degree. The experimental work leading to this report was done *in absentia* at Dartmouth Medical School.

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‡ Fellow of the Jane Coffin Childs Memorial Fund for Medical Research. This investigation has been aided by a grant from the Jane Coffin Childs Memorial Fund for Medical Research.

the New England Nuclear Corp. Polyuridylic acid (poly-U) was a gift of Dr. Severo Ochoa and was also purchased from Miles Laboratories; it possessed an $s_{20,w}$ of 3 S. Creatine phosphate was obtained from California Corp. for Biochemical Research and from Sigma Chemical Co. Creatine kinase was a gift from Dr. Lafayette Noda.[1] The composition of the L-amino acid mixture used was described by Lucas *et al.* (1964). The reagent mixture for glucose determinations, "Calsuls, True Glucose Test," Catalog No. 869004, was a product of the California Corp. for Biochemical Research. All other materials were obtained as previously described (Lucas *et al.*, 1964).

Growth of Cells. S. cerevisiae was grown in 45.5-l. carboys containing 44 l. of culture medium which was stirred vigorously and which was aerated at 5 l./min through a gas dispersion tube. The temperature was maintained at 30 ± 0.1° by means of thermistor-controlled external heating lamps. The medium was inoculated with 200 ml (50 ml in Figure 9) of a pre-inoculum grown well into the stationary phase, cells for the latter being obtained from a slant culture. The composition of the agar slant medium, liquid culture medium, and further details concerning the conditions of cell growth were described previously (Lucas *et al.*, 1964). In most cases, cells were required to be harvested at two or three selected turbidity values (30, 100, and occasionally 280 units as measured in the Klett–Summerson photometer at 420 mμ); these harvests were always obtained from a single batch culture. Experiments in which the turbidity was kept constant for extended periods were performed by employing an arrangement which permitted the continual manual monitoring of the turbidity of the culture and the intermittent addition of fresh sterile medium. At the selected turbidity value, appropriate volumes of the culture were chilled by pouring onto cracked ice, and the cells were harvested immediately. Except as otherwise noted, the procedure for harvesting, washing, and storing the cells was as described previously (Lucas *et al.*, 1964).

Preparation and Incubation of Ribosomes. With the exception of one experiment (Figure 9), ribosomes were prepared by grinding with alumina as described previously (Lucas *et al.*, 1964). Washed ribosomes were prepared as follows. After removal of the initial supernatant fluid, the ribosomal pellet was suspended as usual in Tris–Mg^{2+} buffer (0.05 M Tris, 0.005 M magnesium acetate, pH 7.6 measured at 22°), at a protein concentration of 7.1 mg/ml. The ribosomal suspension was then recentrifuged at 100,000*g* for 2 hr or at 150,000*g* for 1 hr. The supernatant fluid was removed and is referred to as "wash fluid." The washed ribosomal pellet was resuspended in a volume of Tris–Mg^{2+} buffer equal to the volume of wash fluid removed. In the poly-U experiments, spermine was included in the incubation mixture because of its large potentiation of the poly-U stimulation (Bretthauer *et al.*, 1963). For the polysome experiment described in Figure 9, the cell extracts were prepared by grinding shell-frozen yeast with twice its weight of washed sea sand (see Bretthauer *et al.*, 1963) for 2–3 min and then extracting with 0.5 weight of Tris–Mg^{2+} buffer. The extract was centrifuged at 10,000*g* for 10 min and a sample (1.8–2.4 ml) was layered directly onto the sucrose gradient. The use of fresh yeast is less convenient but improves the polysome yield; slow freezing causes its drastic diminution.

Preparation of pH Fraction. The 100,000*g* supernatant fluid was adjusted to pH 5.0 with acetic acid. After standing for 5–10 min, the suspension was centrifuged at 5000*g* for 10 min, and the clear supernatant solution was discarded. The pellet was resuspended in a volume of the previously mentioned Tris–Mg^{2+} buffer equivalent to the original volume of 100,000*g* supernatant fluid. The insoluble material was removed by centrifugation at 15,000*g* for 20 min and the pH 5.0 precipitation procedure was repeated. The pellet was resuspended in a small amount of Tris–Mg^{2+} buffer, insoluble material was again removed, and the protein concentration was adjusted to 4.5–7.5 mg/ml with the buffer.

Density Gradient Centrifugation. Density gradient centrifugations were done in the SW 25.1 rotor of the Spinco ultracentrifuge, equal fractions of approximately 1 ml, numbered from the bottom of the tube, were collected, and 0.1 ml was removed from each fraction for counting. Gradients were linear and were 5–20% sucrose in the experiment described in Figure 6 and 15–30% sucrose in Figures 8 and 9. The time of centrifugation was 150 min. Fractions obtained which were to be resubjected to gradient centrifugation were first appropriately diluted to avoid density inversion.

Determination of Radioactivity. Samples (0.1 ml) were transferred to filter paper disks (Whatman No. 3 MM, 1-in. diameter), and several seconds thereafter the disks were immersed in 5% trichloroacetic acid, washed, and counted in a scintillation counter. When it was desired to count samples larger than 0.1 ml in volume, several disks could be used and placed together in the same scintillation vial. The observed radioactivity is linear with increasing number of up to 4 disks/vial. These and all other operations in this procedure were carried out according to Mans and Novelli (1961) with the exception that it was not found necessary to dry the disks in a stream of hot air prior to their immersion in the trichloroacetic acid. Such drying was found to be hazardous; if carried on too long or at too elevated a temperature, some otherwise soluble nonprotein radioactivity can no longer be removed from the disk. The results of extensive testing of this method under a variety of conditions confirm the results of Mans and Novelli (1961) on the reliability of the method.

Measurement of Q_{O_2}. Harvested cells were washed once with ice-cold water, suspended in 0.05 M potassium phosphate buffer, pH 4.6, 1% in glucose, to give a turbidity (420 mμ) of 114 Klett units. Their oxygen consumption was then measured with the aid of the

[1] We wish to thank Dr. Lafayette Noda for his continued generosity in providing us with supplies of this enzyme.

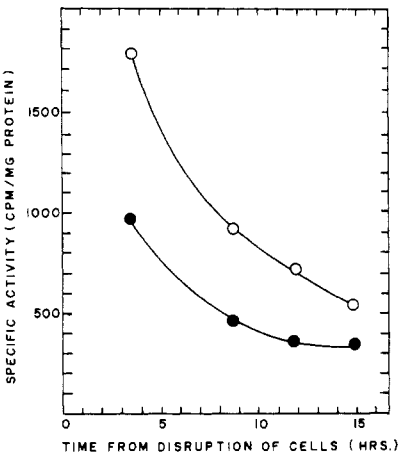

FIGURE 1: Stability of ribosomes stored in the cold. After preparation of the ribosomes, they were suspended in the usual Tris–Mg^{2+} buffer and permitted to stand at about 3°, samples being withdrawn for incubation at appropriate time intervals. The incubation mixture contained 1.25 μmoles of creatine phosphate, 0.25 μmole of adenosine triphosphate (ATP), 0.025 μmole of guanosine triphosphate (GTP),[3] 15 μmoles of KCl, 1 μmole of magnesium acetate, 0.1 μmole of DL-[1-^{14}C]leucine containing 0.4 × 10^6 cpm, 12.5 μg of creatine kinase, 3.75 μmoles of Tris pH 7.6, pH 5 fraction containing 0.25 mg of protein, and ribosome suspension containing 0.38 mg of protein, in a final volume of 0.2 ml. The samples were incubated for 35 min at 30°: O–O–O, OD 100 preparation; ●–●–●, OD 30 preparation.

Clark oxygen electrode[2] as described by Smith and Camerino (1963). Dry weights were obtained by washing the cells twice with water and drying them to constant weight.

Glucose Determination. Glucose was determined on the supernatant fluid, after centrifugation of the cells, by measurement of the reduction of triphosphopyridine nucleotide (TPN^+)[3] at 340 mμ in the presence of hexokinase and glucose 6-phosphate dehydrogenase (see Materials).

Determination of Protein and RNA. Protein determinations were done either by the biuret method (Layne, 1957) or, when small amounts of material were involved, by the method of Lowry *et al.* (1951). Ribosomal RNA was determined after acid hydrolysis by chromatography of the nucleotides (Smith and Markham, 1950; Lucas *et al.*, 1964) or, spectrophotometrically, by the absorbance of a ribosomal suspension at 260 mμ. In the latter case, the extinction coefficient was determined by the method of Smith and Markham (1950); 1 mg/ml of ribosomal RNA gave an absorbance of 22.2.

[2] Thanks are due to Dr. Lucile Smith for valuable advice concerning the use of this instrument.

[3] Abbreviation used in this paper: GTP, guanosine triphosphate; ATP, adenosine triphosphate.

Results

Experiments on Isolated Ribosomes

The primary question asked in this series of experiments is whether the differences in amino acid incorporating activity between ribosomal preparations obtained in different parts of the log phase result simply from a disparity in m-RNA content leading to altered polysome content, or whether the polysomes themselves possess different incorporating activities. (The rate of incorporation of labeled amino acids into ribosomal protein is hereafter referred to as ribosomal activity.) Preliminary to this, a few experiments are reported on some elementary physical and chemical properties of the preparations and on their stability. For convenience, experiments have in most cases been confined to the comparison of two types of ribosomal preparations: one, of low activity, was obtained from cells harvested at a turbidity of 30 Klett units (OD 30 preparation), while the second, of about twofold higher activity, was obtained from cells harvested at a turbidity of 100 Klett units (OD 100 preparation). While the use of ribosomes from cells harvested at turbidities less than 30 Klett units would have resulted in a much greater difference (fourfold) in the incorporating activities of the two preparations under comparison (Lucas *et al.*, 1964), this advantage would have been outweighed by the very small total yield of cells at these low cell densities.

RNA/Protein Ratios and Base Composition of RNA. It can be seen from Table I that the differences in the

TABLE I: Composition and Sedimentation Constants of OD 100 and OD 30 Ribosomal Preparations.

Ribosome Prepn	mg of RNA[a] / mg of Protein	$S_{20,w}$ (S)[b]
OD 30	0.89	80.6
OD 100	0.85	81.4
OD 30 washed	0.94	
OD 100 washed	0.91	

[a] The values reported are the average of determinations made on two preparations of ribosomes prepared from the same yeast culture. [b] These values were obtained at a concentration of ribosomes equivalent to 0.05 mg/ml of ribosomal RNA and were not extrapolated to zero concentration. The ultraviolet optics of the Spinco Model E centrifuge were used.

RNA/protein ratios between the two preparations are small, either before or after washing. Experiments are in progress to determine whether these differences, which can be seen in every preparation, are significant. That no dissimilarity exists in the RNA base composition of the two ribosomal preparations may be inferred from the results of Lucas *et al.* (1964) which show that no

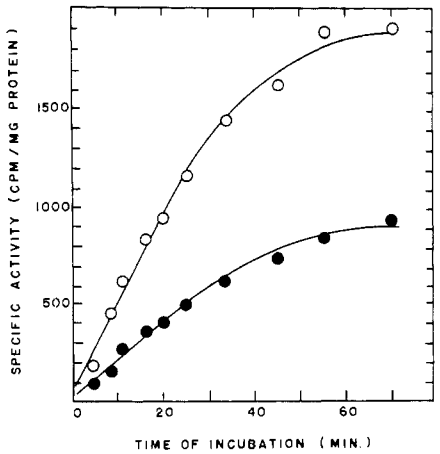

FIGURE 2: Kinetics of incorporation of amino acid into ribosomal protein. A single incubation mixture was made up for each ribosomal preparation and 0.1-ml samples were removed serially at appropriate time intervals. The incubation mixture contained 6.25 μmoles of creatine phosphate, 1.25 μmoles of ATP, 0.125 μmole of GTP, 75 μmoles of KCl, 5 μmoles of magnesium acetate, 0.5 μmole of DL-[1-^{14}C]leucine containing 2 × 10^6 cpm, 62.5 μg of creatine kinase, 25 μmoles of Tris pH 7.6, pH 5 fraction containing 1.3 mg of protein, and ribosome suspension containing 1.56 mg of protein, in a final volume of 1.25 ml. The samples were incubated at 30°: O–O–O, OD 100 preparation; ●–●–●, OD 30 preparation.

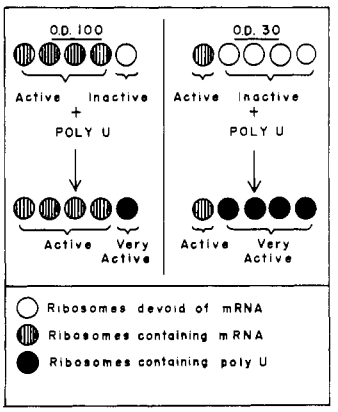

FIGURE 3: Diagram of the use of poly-U as a probe for determining the degree of saturation of ribosomes with endogenous m-RNA.

change occurs in the base ratios of total yeast RNA throughout the log phase.

Sedimentation Analysis. A comparison of the sedimentation constants of the ribosome monomers showed no appreciable difference (Table I). The possibility that the OD 30 preparation is less active because it contains a higher proportion of inactive ribosomal subunits was considered but found to be untenable; there was little indication in either preparation of the presence of such subunits.

Stability of Ribosomes on Standing. Yeast ribosomes lose a major portion of their activity on storage in the cold, unfrozen, for more than 1 day (Lucas, 1963). This raised the possibility that the differences in ribosomal activity between the OD 30 and the OD 100 preparations, routinely assayed about 8–10 hr after the disruption of the cells, resulted from a differential loss in activity during this period. The results of an experiment, shown in Figure 1, in which rapidly prepared and assayed ribosomes are compared show that an appreciable loss in activity occurs in both OD 30 and OD 100 preparations. However, it can also be seen that, at 3.5 hr after cell disruption, the ratio of the activities of the two preparations is 1.8; the ratio remains at this value at 8.3 hr and decreases to 1.5 at 15 hr. Thus, if there is any difference in stability at all, it is evident that time of standing would act to make the ribosomal activities more alike rather than to increase the differences between them.

Stability of Ribosomes during Incubation. The results in Figure 2 show a disparity in both the extent and the initial rates of incorporation. Both preparations maintain a linear rate of incorporation for similar lengths of time; the difference in extent of incorporation is a reflection of their unequal initial rates. Thus, the disparity in activity of the two preparations is evidently not a matter of a difference in the stability of the ribosomal-incorporating system during incubation.

Effect of Addition of Ribosomal Wash Fluid. It is known from earlier studies that yeast ribosomes lose appreciable amounts of their activity on washing but that this activity may be restored to within 10% of the original value by the readdition of the wash fluid (Lucas, 1963). Table II shows the results of a reciprocal cross experiment in which OD 100 and OD 30 ribosomes were incubated with "homologous" and "heterologous" wash fluids. The results show that it makes no difference which wash fluid is used; the OD 100 ribosomes seem to maintain their identity as the more active preparation and the OD 30 the less active. Confirmation of these results was obtained when the two wash fluids were added to unwashed ribosomes (Table II). It appears, then, that the factor which is responsible for the different ribosomal activities is not extractable from the ribosomes by the washing procedure employed. Experiments are in progress to test ribosomes washed by more drastic procedures. It may be noted here that the addition of soluble-RNA (s-RNA) exerts no stimulatory effect on these preparations.

Poly-U as a Probe for m-RNA Content. Any difference between the m-RNA content of the OD 100 and OD 30 ribosomal preparations might result in the observed disparity in their amino acid incorporating activities. The direct quantitative assay of m-RNA in a ribosomal preparation is technically difficult and unreliable for a number of reasons, and therefore an indirect method based on the response of the preparations to poly-U was

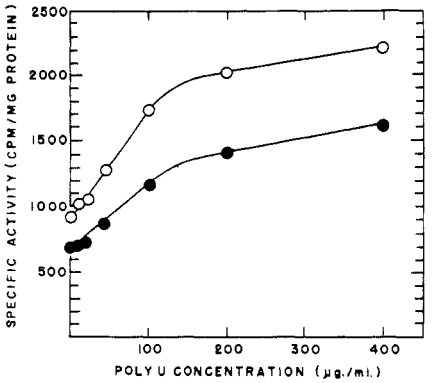

FIGURE 4: The effect of poly-U on the incorporation of phenylalanine by OD 100 and OD 30 ribosome preparations. The incubation mixture contained 1.25 μmoles of creatine phosphate, 0.25 μmole of ATP, 0.025 μmoles of GTP, 15 μmoles of KCl, 0.5 μmole of magnesium acetate, 0.07 μmole of spermine, 0.02 μmole of L-[U-^{14}C]phenylalanine containing 1.0 × 10^6 cpm, 12.5 μg of creatine kinase, 3.75 μmoles of Tris pH 7.6, 0.05 ml of ribosomal suspension containing 0.38 mg of protein, and 0.025 ml of pH 5 fraction containing 0.11 mg of protein, in a final volume of 0.25 ml. Samples were incubated at 30° for 50 min: O–O–O, OD 100 ribosomes; ●–●–●, OD 30 ribosomes.

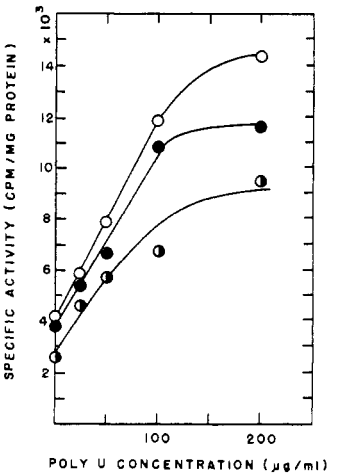

FIGURE 5: The effect of poly-U on the incorporation of phenylalanine by OD 100, OD 30, and OD 280 ribosome preparations. The conditions of the incubation were the same as those described in Figure 4: O–O–O-, OD 100 ribosomes; ●–●–●-, OD 30 ribosomes; ◑–◑–◑-, OD 280 ribosomes.

used. This method is based on the argument that the response of a ribosomal preparation to an artificial messenger (*e.g.*, poly-U) will be inversely related to the amount of natural messenger already present in the ribosomes. The argument is depicted in Figure 3. It is evident from the diagram that the OD 100 preparations comprising a higher proportion of ribosomes containing m-RNA molecules will incorporate amino acids into protein more actively than the OD 30 preparation. However, if poly-U is added and phenylalanine incorporation is measured, then the OD 30 preparation interacting with more poly-U and therefore containing a higher proportion of ribosomes which can make polyphenylalanine would become the more active preparation. A similar argument was employed by Henshaw *et al.* (1963) in an attempt to compare the m-RNA content of microsomes and free ribosomes from liver, and by Florini and Breuer (1965) who compared muscle and liver ribosomes.

The effect of increasing concentrations of poly-U on the activity of OD 30 and OD 100 ribosomes is shown in Figure 4. It may be seen that, at every concentration of poly-U, the OD 100 preparation remains the more active one. If there is any change at all in the relationship between the two activities, the OD 100 preparation is stimulated slightly more by poly-U than is the OD 30. These results suggest that the OD 30 preparation does not contain more "empty" ribosomes than does the OD 100. A similar conclusion can be drawn from the results of a poly-U experiment (Figure 5) in which OD 100 ribosomes were compared with OD 280 ribosomes.

TABLE II: Amino Acid Incorporation by Ribosomes Incubated with Homologous and Heterologous Ribosomal Wash Fluids.[a]

Type of Ribosome Added	Specific Activity of Protein (cpm/mg of Protein) Type of Ribosomal Wash Fluid Added[b] No addition	OD 100	OD 30
Washed ribosomes			
OD 100	780	1265	1165
OD 30	630	800	915
Unwashed ribosomes			
OD 100	1420	1615	1690
OD 30	900	995	1005

[a] The incubation mixture contained 1.25 μmoles of creatine phosphate, 12.5 μg of creatine kinase, 0.25 μmole of ATP, 0.025 μmole of GTP, 15 μmoles of KCl, 0.5 μmole of magnesium acetate, 0.07 μmole of spermine, 10 μmoles of Tris pH 7.6, 0.1 μmole of DL-[1-^{14}C]-leucine containing 0.4 × 10^6 cpm, 0.25 ml of washed pH 5 fraction containing 0.11 mg of protein, and 0.05 ml of ribosomal suspension containing 0.38 mg of protein, in a final volume of 0.25 ml. The incubation was carried out at 30° for 50 min. [b] When used, 0.05 ml of wash fluid was added and corresponding amounts of Tris and Mg^{2+} were omitted from the basic incubation mixture.

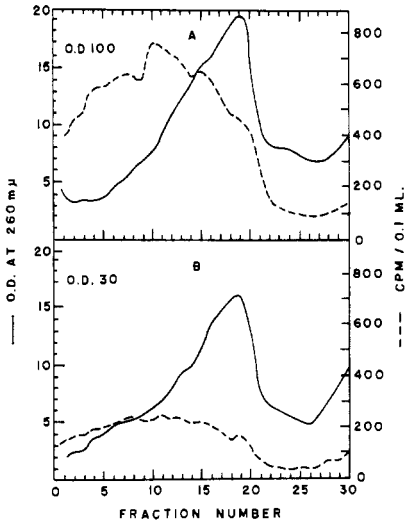

FIGURE 6: Sucrose density gradient centrifugation of incubated ribosomes. The incubation mixture contained 7.5 μmoles of creatine phosphate, 75 μg of creatine kinase, 1.5 μmoles of ATP, 0.15 μmole of GTP, 90 μmoles of KCl, 30 μmoles of Tris pH 7.6, 6.75 μmoles of magnesium acetate, 0.0022 μmole of L-[U-^{14}C]-phenylalanine containing 0.8 × 10^6 cpm, 0.065 μmole of L-[U-^{14}C]alanine containing 0.8 × 10^6 cpm, 0.0030 μmole of L-[U-^{14}C]leucine containing 0.8 × 10^6 cpm, 0.25 ml of pH 5 fraction containing 0.75 mg of protein, and 0.50 ml of ribosomal suspension containing 5.9 mg of protein, in a final volume of 1.5 ml. The incubation was carried out for 10 min at 30°: curve A, OD 100 ribosomes; curve B, OD 30 ribosomes.

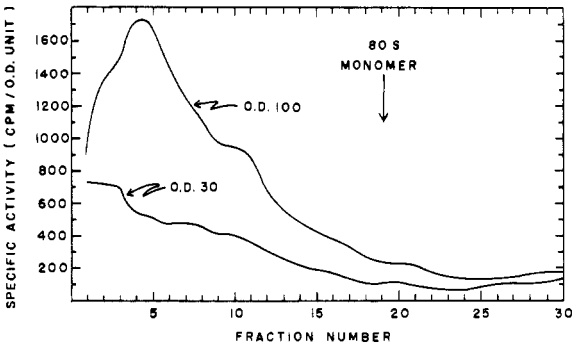

FIGURE 7: Specific activity of the fractions obtained from incubated ribosomes after density gradient centrifugation. These values are computed from the data in Figure 6. An OD unit is defined as the milliliters of radioactive fraction counted multiplied by the OD of the fraction.

TABLE III: Comparison of Leucine and Poly-U Stimulated Phenylalanine Incorporation into OD 100 and OD 30 Ribosomes.[a]

Ribosome Prepn	Specific Activity of OD 30 Ribosomes Divided by Specific Activity of OD 100 Ribosomes		
	Endogenous Incorporation		Poly-U Stimulated Phenylalanine Incorporation[b]
	Leucine	Phenylalanine	
Unwashed	0.63	0.67	0.64
Washed	0.81		0.86

[a] The incubation mixture for leucine incorporation contained 1.25 μmoles of creatine phosphate, 0.25 μmole of ATP, 0.025 μmole of GTP, 15 μmoles of KCl, 1.5 μmoles of magnesium acetate, 0.1 μmole of DL-[1-^{14}C]leucine containing 0.4 × 10^6 cpm, 12.5 μg of creatine kinase, 8.75 μmoles of Tris pH 7.6, 0.025 ml of pH 5 fraction containing 0.26 mg of protein, and 0.05 ml of ribosome suspension containing 0.32 mg of protein in a final volume of 0.25 ml. The incubation mixture for phenylalanine incorporation is given in Figure 4. The incubations were carried out for 60 min at 30°.
[b] The incubation mixture contained 20 μg of poly-U.

(The latter are sluggish ribosomes obtained late in the log phase.)

An occasional batch culture of yeast would, for an unknown reason, yield OD 100 and OD 30 ribosomes of identical or nearly identical endogenous activity. When poly U was added to such preparations, however, the customary qualitative differences in activity appeared (Figure 5) although the quantitative differences between the two preparations were not as large as usual.

The conclusion reached above is reinforced by the results given in Table III in which the activities of the two ribosome preparations are assayed in two ways, the endogenous rate by leucine incorporation and the rate when artificial messenger (poly-U) is added by phenylalanine incorporation. The results show again that the addition of poly-U does not change the relative rates of incorporation by the two preparations, either when washed or unwashed ribosomes are used. These results also show that the greater activity of the OD 100 ribosomes is independent of the amino acid used to assay the activity.

Thus, while the activity of a ribosomal preparation is undoubtedly dependent on the availability of m-RNA, the results suggest that the difference between the activity of the OD 100 and the OD 30 ribosomes is not related to their content of m-RNA and that therefore there is yet another factor associated with the ribosome which modulates its activity.

Polysome Content of the Ribosomal Preparations. It is now recognized from experiments on a number of bacterial and mammalian systems that the polysome is

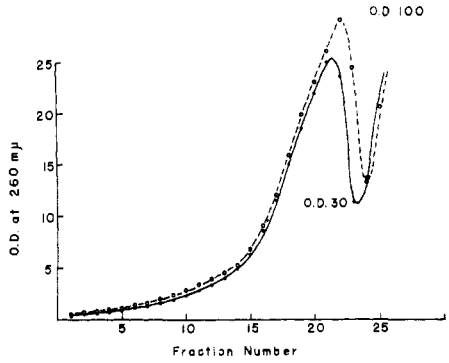

FIGURE 8: Isolation of polysomes by sucrose gradient centrifugation. An extract of sand-ground cells was centrifuged at 10,000*g* for 10 min and the supernatant fluid was placed on the gradient. All other conditions are as described in Experimental Procedure.

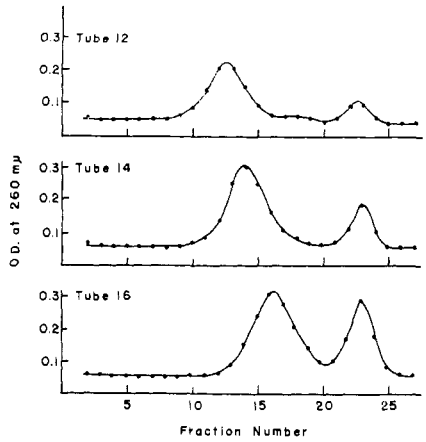

FIGURE 9: Recentrifugation in sucrose gradients of polysome fractions obtained as described in Figure 8. Immediately after collecting the fractions from the original gradient (Figure 9), 1-ml samples of the appropriate fractions (in 22–25% sucrose) were diluted with equal parts of the Tris–Mg^{2+} buffer and were layered onto a second gradient which was run immediately. All other conditions were identical with those described in Figure 8 and in Experimental Procedure.

the ribosomal form most active in protein synthesis, and this also appears to be the case in yeast (Marcus *et al.*, 1963). The possibility is thus raised that the difference in activity between OD 100 and OD 30 ribosomes is related to the presence in the ribosomal preparations of different amounts of these active polymeric forms. To test this possibility, incubated ribosomes were subjected to sucrose density gradient centrifugation in order to examine the polysome region. The results are shown in Figure 6.

If the amount of polysome material (say, in tubes 1–10) is now computed for each preparation (by determination of the areas under the curves), it is seen that there is little difference between them, the ratio for the two preparations (OD 100/OD 30) being 1.15. The actual difference between the two preparations is even less than this ratio would indicate since the ratio needs to be corrected downward 10–15% because a slightly greater amount of total material was present in the OD 100 gradient. Thus, it does not appear that the two preparations differ in their polysome content to an appreciable extent. If the data in Figure 6 are used to compute specific activities, it appears (Figure 7) that the OD 100 polysomes are considerably more active than are the OD 30.

This interpretation of the results is valid only if the 260-mμ-absorbing material in the polysome region accurately reflects the amount of polysomes present and is not partly the result of "spillover" into this region of some of the large amount of material present in the nearby 80 S peak. While this view is not considered likely for regions very far removed from the 80 S peak, it was nevertheless felt necessary to design an experiment which would reduce possible contamination. To this end, ribosomes were prepared from sand-ground cells, a procedure which increases the yield of polysomes. In addition, the experiment was performed differently in that the polysome fractions were first isolated from the gradient and then incubated. Further, the polysome content of the isolated fractions was determined by rerunning them on a fresh gradient.

The results of the density gradient runs are shown in Figure 8. It can be seen that little difference is discernible between the polysome content [100% × polysomes/(polysomes + monomer)] of the OD 100 and the OD 30 preparations; 19.5% for the former, 20.0% for the latter. (Other experiments in which the polysome content was of the order of 30% gave similar results; no differences could be found between the polysome content of the two preparations.) Control experiments in which the cell extract was incubated with RNAase showed no polysomes on density gradient centrifugation. Fractions 14 and 15 were selected for incubation and the results, shown in Table IV, indicate that the OD 100 polysomes are 40–50% more active that the OD 30. Since the preparation of these polysomes by gradient centrifugation is at least the equivalent of the washing procedure employed in the preparation of washed ribosomes as far as the removal of soluble material is concerned, it would be expected that the addition of wash fluid to the polysomes would further increase the difference between the OD 30 and OD 100 preparations (see Table II). Such experiments are in progress.

Many experiments have been run to assess the polymer and monomer content of some of the fractions obtained from gradient runs such as that described in Figure 8. The results are surprisingly uniform from experiment to experiment. Gradient runs of a number of such fractions are shown in Figure 9. Measurement of the areas under the curves indicates that the monomer contamination of tubes 12, 14, and 16 is 14, 21, and 35%, respectively. The values for contamination by

TABLE IV: Amino Acid Incorporation by Polysomes Obtained from Density Gradient Centrifugation of Sand-Ground Cells.[a]

Sucrose Gradient Fraction No.	Incubation Time (min)	Specific Activity (cpm/mg of ribosomal RNA) Ribosome Preparation OD 100	OD 30
14	15	3280	2320
	30	5700	4350
15	15	3100	2180
	30	5450	3860

[a] The incubation mixture contained 5 μmoles of creatine phosphate, 1 μmole of ATP, 0.1 μmole of GTP, 60 μmoles of KCl, 3.75 μmoles of magnesium acetate, 0.12 μmole of DL-[1-^{14}C]leucine containing 3.3 × 10^6 cpm, 50 μg of creatine kinase, 37.5 μmoles of Tris pH 7.6, 0.15 ml of pH 5 fraction[b] containing 0.45 mg of protein, 0.6 ml of polysomes in 22% sucrose containing 0.23 mg of RNA (fraction 14) or 0.29 mg of RNA (fraction 15), in a final volume of 1.12 ml. The incubation temperature was 30°. [b] The pH 5 fraction was prepared from the pooled post-ribosomal supernatant fluids of alumina-ground OD 30 and OD 100 cells.

monomer are maximal values since it is probable that some conversion of polymer to monomer occurs during recentrifugation.

When the contents of fraction 14 were subjected to analytical ultracentrifugation with the aid of the ultraviolet optics, values of $s_{20,w}$ of 78 and 236 S were obtained for the two peaks. According to Wettstein *et al.* (1963), the latter value corresponds to a polysome hexamer. It thus appears from the evidence at hand that the polysome content of OD 100 and OD 30 preparations is the same but that polysomes from the two preparations possess different incorporating activities.

Experiments on Growing Yeast Cells

Effect of Modifications of the Yeast Growth Cycle on Activity of Isolated Ribosomes. The variation during the log phase of a cellular parameter, in this case ribosomal activity, could arise in response to either an internal or an external stimulus to the cell. An external stimulus might occur as a result of quantitative or qualitative changes in the composition of the medium during culture growth. Stimuli from within the cell might occur from many sources. One possibility might be that although the minimal conditions necessary for cell division may have been achieved during the lag phase, thus permitting the onset of logarithmic growth, conditions at this point may not yet be optimal for all of the activities of the cell which involve protein synthesis. The fulfillment of these conditions could take a few generations.

The effects of such internal stimuli would be expected to be either time dependent or dependent upon the number of cell divisions since lag phase. On the other hand, effects arising solely from changes in the medium should disappear if the composition of the medium is held constant, for example, by growing cells in the log phase under steady-state conditions. An experiment was designed to distinguish between these possibilities. Cells were grown into the log phase until the turbidity of the culture reached about 85 Klett units (a point prior to that at which the peak of *in vitro* ribosomal activity is reached). The cell density was then kept approximately constant over about three generations by the continual addition of fresh medium in small increments. (This period of time would have brought the culture into the stationary phase in batch growth.) The growth of the culture was then permitted to proceed as usual into the stationary phase. Samples of culture were removed throughout the growth period and ribosomal activity was examined as usual. If the "internal stimulus" hypothesis is correct, no difference should be observed between the results of this experiment and the usual batch culture experiment. However, if changes in the medium are responsible, ribosomal activity should rise until steady-state conditions have been applied, at which time increase in activity should cease and constant activity be maintained. When the culture is again allowed to grow as usual, ribosomal activity should show an increase once more until the usual peak at OD 100, at which point the customary decrease in activity should be observed.

A comparison of the results of this experiment (Figure 10B) with those obtained from the usual batch growth experiment (Figure 10A, taken from Lucas *et al.*, 1964) clearly shows that the latter predictions are borne out. It thus appears that the events leading to the variation in ribosomal activity are caused by some change in the culture medium.

These conclusions are reinforced by the results of an experiment[4] in which the lag phase was completely eliminated from the growth cycle. This was accomplished by growing the inoculum into the log phase under the same conditions as that of the batch culture until a turbidity of 100 Klett units was reached (at which point ribosomal activity is at a maximum). An amount of this OD 100 cell suspension, containing the same total number of cells as the usual inoculum used, was transferred to fresh medium previously brought to the proper temperature, and the remainder of the experiment was carried out as usual. The results showed that the customary peak of ribosomal activity was obtained, indicating that neither the time period nor the number of cell divisions since lag phase is a factor in the change in ribosomal activity.

Changes in the Composition of the Medium. It can be seen from the results in Figure 11 that continuous

[4] This experiment was performed by Mr. Leonce G. Evans under the auspices of the Summer Research Program sponsored by the U. S. Public Health Service (grant 5T5-GM-63).

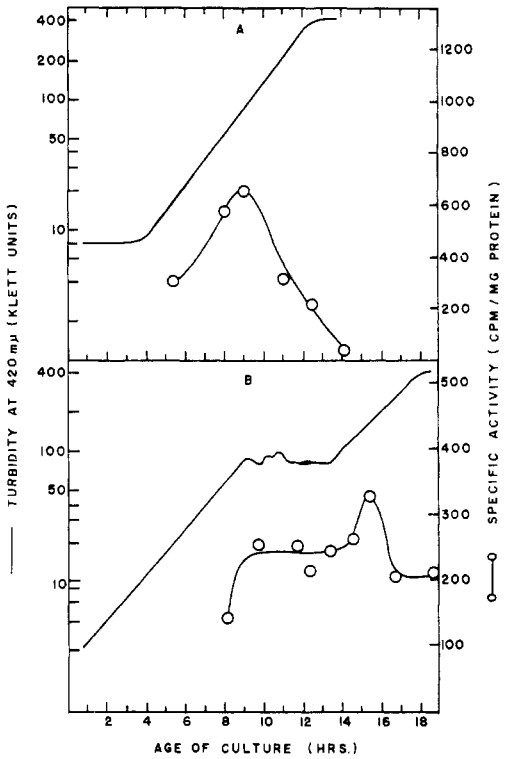

FIGURE 10: Effect on ribosomal activity of maintaining the cell culture at constant turbidity. The details of the experimental procedure are given in the text. The incubation mixture for Figure 10A is described by Lucas *et al.* (1964). The incubation mixture for Figure 10B contained 5 μmoles of creatine phosphate, 50 μg of creatine kinase, 0.1 μmole of ATP, 60 μmoles of KCl, 65 μmoles of Tris pH 7.6, 5 μmoles of magnesium acetate, 0.5 μmole of DL-[1-^{14}C]leucine containing 1.6 × 10^6 cpm, 100,000*g* supernatant fluid containing 1.2 mg of protein, and ribosomal suspension containing 1.7 mg of protein, in a final volume of 1 ml. The incubation was carried out for 30 min at 30°. The protein was washed and counted as described by Lucas *et al.* (1946). One specific activity value obtained in early log phase was so obviously spurious that it was omitted. The slight variations in turbidity values in the "constant turbidity" portion of the curve were unintentional and result from the use of a manually operated turbidostat: ———, turbidity; O—O, specific activity. This experiment was done before it was discovered that this concentration of Tris buffer is somewhat inhibitory (Dietz, 1965).

changes in the composition of the medium do take place. At about mid-log phase, the glucose level begins to fall precipitously and a sudden drop in pH at this point can also be observed.

Changes in the Metabolism of the Cell. Figure 12 illustrates the respiratory capacity of the cell at different points in the log phase. It can be seen that after a period of increasing Q_{O_2} a sharp drop in respiratory capacity takes place at about mid-log phase.

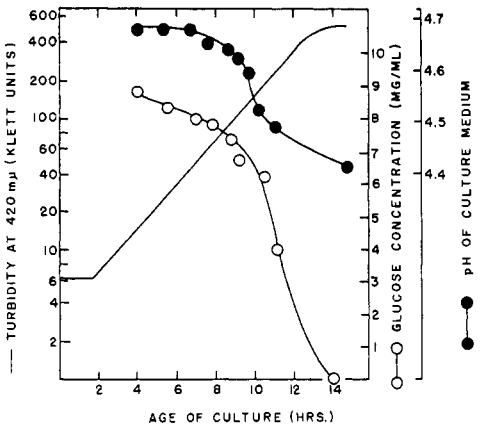

FIGURE 11: Changes in the composition of the medium during the log phase.

Discussion

When yeast cells are harvested at different points in the exponential phase of growth and ribosomes are prepared from these harvests, the various ribosomal preparations are found to possess different amino acid incorporating activities (Lucas *et al.*, 1964). The maximum activity is always found in ribosomes obtained from cells harvested at mid-log phase, the activity rising in early log phase and decreasing in late log phase. The primary problem considered here is whether the alteration in ribosomal activity is merely a reflection of the m-RNA content and therefore the polysome content of the different preparations, or whether the differences in activity result from inherent differences in the ribosomes and polysomes themselves.

The results fail to demonstrate differences in the content of m-RNA in active and sluggish ribosomal preparations. Further, evidence is presented that the polysome content of the two preparations is the same, even with respect to size distribution. More interesting, however, is the observation that when polysomes from active and sluggish ribosomal preparations are compared, they themselves are correspondingly active and sluggish.

It is not at all clear at the present time what factors are associated with the polysome which might exert control over its activity. The few exploratory experiments reported here, performed in order to gain some clue to the difference between the active and sluggish preparations, were essentially negative; no differences could be found in sedimentation constants, RNA base ratios (Lucas *et al.*, 1964), stability to standing or to incubation, or effect of washing or readdition of the wash fluid. Whether or not the small difference in RNA/protein ratio is significant is under investigation, along with a variety of functional properties of the ribosome. It is worth pointing out that Hoagland *et al.* (1964), in their comparison of microsomes from regenerating and normal livers, have obtained evidence for the existence

of an inhibitor associated with the less active microsomes from normal liver.

The sequence of events during the growth cycle which leads to an alteration of ribosomal activity and to an alteration in the cellular content of ribosomes is not understood. Our attempts to study these events experimentally cannot be regarded as more than preliminary. It is clear, however, that not only can ribosomal changes take place during the log phase, but also that, from these experiments and those of others (see following discussion), a number of other profound changes can occur in the metabolism, structure, and enzyme complement of exponentially multiplying cells without affecting their rate of multiplication. It is worth noting at this point that ribosomal activity measured *in vitro* may not be a true reflection of the rate of protein synthesis in the intact cell.

The role of glucose in controlling the respiratory capacity of the yeast cell was clearly demonstrated by Ephrussi *et al.* (1956), first in experiments such as those reported here in which the Q_{O_2} and other metabolic parameters were followed during log growth in batch culture and then in experiments in which logarithmic growth was maintained in essentially continuous culture. In the batch culture experiments, the glucose concentration falls with time, and when Q_{O_2} measurements are made on cells harvested at various points in the growth curve, the respiratory capacity of the cells was found to vary throughout the log phase in a continuous but complex manner. In the continuous culture experiments, the effect on various metabolic parameters of different glucose levels was investigated. It became evident from both types of experiments that for every glucose level in the growth medium there exists a corresponding set of values for the Q_{O_2}. It was further evident that an inverse relationship exists between the glucose concentration and the Q_{O_2}; that in cells grown at high glucose levels, respiration seems to be inhibited. Accompanying this inhibition of respiration is a depression in the synthesis of mitochondria and of the cytochromes, and a stimulation of the capacity for aerobic glycolysis. These findings may well be part of the well-known phenomenon of catabolite repression (Magasanik, 1961).

Although the conditions under which our yeast was grown are not precisely those of Ephrussi *et al.* (1956), particularly with respect to important aspects such as extent of aeration of the growth medium and Q_{O_2} of the inoculum cells, the glucose concentrations used are similar and it is probable that this glucose effect is at least partly responsible for some of the variation in Q_{O_2} which we observe. Another factor at work may be the beginning of an oxygen deprivation of the medium at cell densities such as those which occur around mid-log phase, a condition difficult to avoid except by special means of aeration.[5] Such a deprivation could result in the sharp decrease in Q_{O_2} seen around mid-log phase. It is evident that this sharp dip in Q_{O_2} occurs at about the same point in the growth curve as that at which ribosomal activity starts to fall.

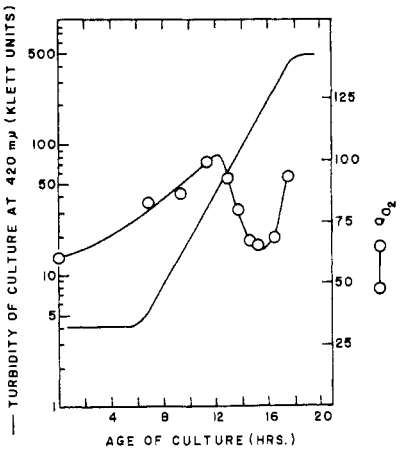

FIGURE 12: Changes in the respiratory capacity of the yeast cell during the log phase.

While a number of effects are taking place concomitantly with changes in ribosomal activity, it is not yet possible to assign a direct cause to these changes. The results show that, first, significant alterations take place during the log phase in the composition of the growth medium, at least with respect to the fall in pH and in the concentration of glucose, and second, that the changes in ribosomal activity are somehow brought about by some change in the composition of the medium. We have also seen from the previous discussion that a fall in glucose concentration is capable of bringing about profound changes in metabolic, enzymatic, and structural parameters of the cell. However, it is not possible to decide at present whether a causal relationship exists between the changes in these metabolic parameters and the altered activity (and cellular content) of ribosomes or whether both phenomena independently result from changes in the medium.

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Heterogeneity of Rabbit γ-Globulin with Respect to Cleavage by Papain*

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ABSTRACT: When γ-globulin from an immunized rabbit was treated with papain for short periods of time, three fractions were recovered by sieving through Sephadex G-200. These had sedimentation coefficients of 3.6, 5.0, and 6.9 S. The 5.0-S fraction is an intermediate in the degradation which, based upon immunologic characterization, appears to consist of one fragment I or II and one fragment III. It lacks the ability to precipitate with antigen but specifically coprecipitates in the presence of undegraded antibody and antigen rather than producing inhibition as do the univalent 3.6-S antibody fragments. The 5.0-S intermediate is fully as active as 7-S antibody in eliciting passive cutaneous anaphylaxis in guinea pigs as only 0.025 μg of either antibody gave distinctly positive reactions. The residual 6.9-S fraction, upon redigestion, was more resistant to the action of papain than the starting material. This resistance did not seem to be related to the presence of fragments I or II in the molecule, but the resistant fraction possessed more hexose than the over-all γ-globulin. The additional hexose was associated with fragment I of the papain-digested molecule. The fragment of H polypeptide chain which is a component of the 5.0-S intermediate was isolated and was found to possess half of the hexose of fragment III, suggesting that the carbohydrate may be symmetrically distributed between the two H polypeptide chains of the γ-globulin molecule.

Rabbit γ-globulin is split by the proteolytic enzyme papain into functionally distinct fragments which have been designated I (or II) and III (Porter, 1959). Fragment I (or II) is composed of an L[1] polypeptide chain, which has a molecular weight of 20,000–25,000, and about half of an H[1] polypeptide chain. The intact H chain has a molecular weight of 50,000–55,000 (Fleischman *et al.*, 1962). Fragment III is composed of the remaining halves of two H chains. These elements fully account for the four polypeptide chains, two of each type, of which native γ-globulin is composed (Fleischman *et al.*, 1963).

During the course of investigations relating to the electrophoretic heterogeneity of fragment III (Paraskevas and Goodman, 1964), γ-globulin was exposed to papain for abbreviated periods of time, the reaction being aborted by addition of iodoacetamide in excess. The mixture was passed through Sephadex G-200 in order to resolve the digestion products from residual undegraded γ-globulin. Three protein peaks were obtained rather than the two which were anticipated. In addition to fractions with s_{20} values of 6.9 and 3.6 S, corresponding to native γ-globulin and its degraded products, respectively, a fraction with an s_{20} value of 5.0 appeared. This intermediate in the conversion of 6.9-S γ-globulin to 3.6-S fragments has recently been reported elsewhere and was characterized as consisting of one fragment I and one fragment III by means of peptide profiles (Nelson, 1964).

* From the Department of Microbiology, University of California, School of Medicine, San Francisco, Calif. *Received April 28, 1965; revised June 29, 1965.* Supported by grants from the National Institutes of Health (AI 05664) and the Breon Fund of the Committee on Research of the University of California.

[1] An alternate nomenclature in which the designations H and L are replaced by A and B, respectively, is also in use.