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A Cell-free Amino Acid-incorporating System from *Saccharomyces cerevisiae*. Variation in Ribosomal Activity and in RNA Synthesis during Logarithmic Growth*

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A cell-free ribosomal system has been obtained from *S. cerevisiae* by lysis of protoplasts and by grinding the cells with alumina. The ribosomes can incorporate labeled amino acids added either as free amino acids or as aminoacyl-s-RNA and the properties of both types of system have been summarized. The amino acid–incorporating activity of the system varies during the logarithmic phase of growth. The ribosomes are responsible for this variation. Paralleling this change in activity is a change in the cellular content of ribosomes and in the rate of synthesis of ^{32}P-pulse-labeled RNA, whose base composition points to an identity with messenger RNA, at least in part. This greatly increased capacity for protein synthesis does not appear to be reflected in an actual increase in the protein content of the intact cell. A beginning has been made in seeking the biochemical factors associated with the ribosome which cause the variation in its activity. The base composition of the RNA does not appear to play such a role as it does not vary throughout the log phase. In view of such variation in messenger RNA synthesis, the ribosomal content of this RNA is being investigated.

Yeast offers a number of potential advantages in studies on protein synthesis. Its amino acid–activating enzymes, s-RNA, and ribonucleoprotein particles have been isolated and extensively studied. In addition, a large number of highly purified and well-characterized proteins, some of which are inducible, have been isolated from this microorganism, thus presenting a wide range of choice for the study of the biosynthesis of a specific protein in a cell-free system. Because an active cell-free preparation from yeast was not available when this work was begun, our efforts were directed toward the development of such a system. This communication describes the isolation of active ribosomes from *S. cerevisiae*, briefly summarizes the properties of the system, and reports on the following unexpected finding.

Early in these studies, it was noted that the activity of the system varied considerably with the batch of yeast used, and a tentative correlation began to appear between the extent of this activity and the age of the yeast culture from which the cell-free system had been prepared. Inasmuch as the cells were always harvested at some point in the log phase, this meant that the activity of the cell-free system and therefore the potential capacity of the cell for protein synthesis was changing during the log phase. A variation in so fundamental a property during a period of growth in which the cells are reproducing at a constant rate makes this phenomenon one of particular interest in terms of the regulatory mechanisms of the cell. The further finding, that the variation in activity of the cell-free system could be attributed to the ribosomes themselves, raises the interesting question of the factor or factors associated with the ribosome which regulates this activity. The changes observed in ribosomal activity are accompanied by parallel changes in the RNA content of the cell and in the rate of synthesis of what is probably messenger RNA. Work is in progress to elucidate the mechanisms involved.

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Experimental Procedure

Materials.—Phosphoenolpyruvate, pyruvate kinase, GTP, ATP, CTP, spermine, GSH, puromycin, and unlabeled amino acids were obtained from the California Corp. for Biochemical Research, [1-^{14}C]leucine from the New England Nuclear Corp., vitamins from Nutritional Biochemicals Corp., agar, bactopeptone, and yeast extract from Difco Laboratories, and RNase and DNase from Worthington Biochemical Corp. The amino acid mixture used in these experiments consisted of the stated amounts of glycine, L-alanine, L-serine, L-threonine, L-isoleucine, L-valine, L-glutamic acid, L-aspartic acid, L-lysine, L-arginine, L-histidine, L-methionine, L-cysteine, L-proline, L-tryptophan, L-phenylalanine, L-tyrosine, L-glutamine, and L-asparagine. [P^{32}]orthophosphate was obtained carrier-free from the Union Carbon and Carbide Co., Oak Ridge,

Tenn. It was used without further purification. Chromatograms were printed with the aid of the photographic paper recommended by Lipkin *et al.* (1959).[1]

Growth of Cells.—The yeast used in these experiments was *Saccharomyces cerevisiae*, strain SLK.[2] It was maintained on slants of agar which were 2% in bactopeptone, 2% in glucose, and 1% in yeast extract. The synthetic culture medium was that described by Halvorson (1958) except that the glucose concentration was reduced to 1%. The vitamin mixture contained per ml: 0.001 mg biotin, 0.3 mg calcium pantothenate, 0.3 mg folic acid, 3.3 mg inositol, 3.3 mg pyridoxine, 3.3 mg nicotinic acid, 3.3 mg *p*-aminobenzoic acid, and 3.3 mg thiamine. The mineral mixture consisted of, per ml: 0.5 mg each of $FeSO_4$, $ZnSO_4$, $MnSO_4$, $CuSO_4$, and 0.001 mmole H_2SO_4.

The yeast was grown in 5- or 12-gallon carboys at 30° with vigorous stirring and aeration, the growth medium having been inoculated with an aqueous suspension of cells obtained directly from slant cultures. Later experiments indicated that the use of a preinoculum, prepared by adding one loopful of yeast from a slant culture to 100 ml of synthetic medium and permitting the yeast to grow at 30° with shaking until the cells were well beyond the logarithmic phase of growth, did not give results in conflict with those to be reported here. At appropriate time intervals, samples were obtained from the growing culture; the volumes removed were such that the total number of cells in each sample was the same. Occasionally, when small amounts of cells were needed, they were grown in Erlenmeyer flasks with continuous shaking. The growth of the culture was followed by measuring its turbidity in the Klett-Summerson photometer using the 420-mμ filter. Values for the turbidity so obtained are given in Klett units. For the investigation of the general properties of the system, the yeast was always harvested at a turbidity of 100 Klett units.

After removal of the appropriate volume of culture, it was cooled rapidly by the addition of ice and was centrifuged at 11,500 rpm with the aid of a Lourdes continuous-flow rotor. The cells were then washed twice with glass-distilled water and stored at −20°.

Preparation and Incubation of Ribosomes.—The frozen cells were ground in the cold in a precooled (−20°) mortar with twice their weight of levigated alumina. When the material reached the consistency of a soft paste (about 10 minutes), it was suspended in a volume of Tris-Mg buffer (0.05 M in Tris and 0.005 M in magnesium acetate) equivalent to three times the weight of the cells. After centrifugation of the suspension at 10,000 × *g* for 15 minutes, and of the resulting supernatant fluid at 30,000 × *g* for 20 minutes in the Spinco Model L ultracentrifuge, the ribosomes were sedimented at 100,000 × *g* for 2 hours. The 100,000 × *g* supernatant fluid was decanted and the pellet was resuspended in the Trig-Mg buffer at a concentration of 7.1 mg protein/ml. Washed ribosomes were prepared by resuspending the pellet in a volume of the buffer equal to the original volume of the suspension, centrifuging at 10,000 × *g* for 10 minutes, discarding the sediment, and centrifuging the supernatant fluid (ribosomal washings) at 100,000 × *g* for 2 hours to resediment the ribosomes. All final concentrations of Mg^{2+} given have been corrected for the amount of this ion introduced by the ribosomal suspension and the *p*H 5 fraction. The reaction was stopped by the addition of trichloroacetic acid, and the protein was washed and plated and radioactivity was counted as described by McLean *et al.* (1958).

Protein Determinations.—In the course of most experiments (where speed of operation was desirable), the concentration of protein in the ribosome, *p*H 5, and in other preparations was determined turbidimetrically (Stadtman *et al.*, 1951). However, this assay method was calibrated against the biuret method (Layne, 1957) and results are expressed in terms of actual protein concentration. The biuret method was also used to measure the cellular-protein content.

Routine Determination of RNA.—For routine determination of RNA, the cells were first washed twice with water, then extracted three times with ethanol at −10° to −20° and then with 0.6 M perchloric acid at 0° to −3° until no further 260 mμ-absorbing material was present in the extracts. The residue was then incubated with 3 ml of 1 N KOH for 20 hours at 37°. After the addition of 0.5 ml of 70% perchloric acid, the material was permitted to stand for 90 minutes at 0°, and was then centrifuged in the cold. The absorbancy at 260 mμ of the supernatant fluid was then measured and the nucleotide content of the solution was calculated using an A_M of 10,800 (Ogur and Rosen, 1950). Experience with this method indicates that when it is used on complex mixtures the results obtained are somewhat higher than those obtained by the more accurate method of quantitative analysis of the bases. It has nonetheless proved useful because of its rapidity, particularly where many points were desired and absolute values for RNA were not required.

Determination of Base Composition of RNA.—After extraction of the cells with ethanol and perchloric acid as described in the previous section, hydrolysis was carried out in 0.3 N KOH at 32° for 21 hours. The solution was chilled to 0° and ice-cold perchloric acid was added to a final concentration of 0.6 M. After standing for a time at 0°, the precipitate was removed by centrifugation in the cold and washed once with a small volume of ice-cold water, and the supernatant fluids were combined. The solution was then brought to *p*H 7 with KOH, a drop of glacial acetic acid was added, and, after standing at 0° for a time, the $KClO_4$ was removed by centrifugation. The sediment was washed once with water and the combined supernatant fluids were chromatographed in the two-dimensional system of Lipschitz and Chargaff (1960). Occasionally, separation of the spots was found to be incomplete and chromatography in the first dimension was repeated.

Spots were located by printing the chromatograms on photographic paper with the aid of a monochromatic-ultraviolet-light source (Lipkin *et al.*, 1959), were then cut out along with nearby blank spots, and were eluted with 0.1 N HCl in the case of the pyrimidine nucleotides while 0.1 N NH_4OH was used for the purine nucleotides. The concentration of the nucleotides was calculated using the A_M values given by Beaven *et al.* (1955).

Pulse-labeling of RNA with ^{32}P.—Yeast was grown as described earlier, cells being harvested at the time intervals indicated. The cells were washed twice with ice-cold low-phosphate medium (identical to the growth medium except that the phosphate concentration was lowered to 0.002 M, necessitating adjustment of the *p*H to that of the growth medium [*p*H 4.9]). An amount of the washed cells was then resuspended in 25 ml of the cold medium such that the final turbidity of the suspension was 200 Klett units (420 mμ). The samples were then rapidly warmed to 30°, 0.5 ml of

[1] The paper (Portagraph G 91) and developer (G 45) are now obtainable from Transcopy, Inc., Newton, N. J.

[2] We wish to thank Dr. Richard Morgan for the yeast culture and for supplying the composition of the vitamin mixture.

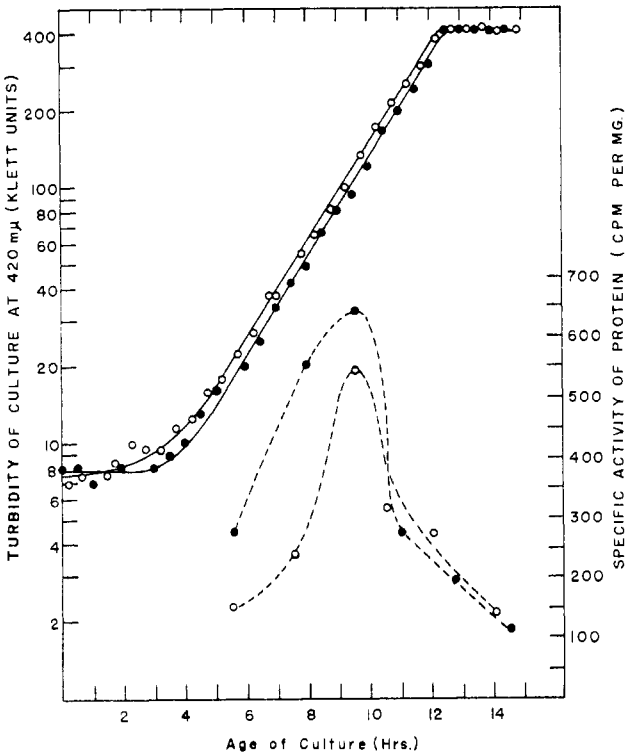

FIG. 1.—The effect of the age of the culture on the ability of the cell-free system to effect the incorporation of [1-^{14}C]leucine into protein. The curves represent the results of two independent experiments carried out under similar conditions. Yeast was grown as described earlier, and batches of cells were harvested at the times indicated by the points on the specific-activity curves. A cell-free system was obtained from each batch of cells and was incubated at 30° for 30 minutes. The incubation mixture contained 1 μmole ATP, 5 μmoles phosphoenolpyruvate, 30 μg pyruvate kinase, 0.05 μmole GTP, 60 μmoles KCl, 0.03 μmole L-[1-^{14}C]leucine containing 8 × 10^5 cpm, 25 μmoles Tris buffer, *p*H 7.6, 2.5 μmoles magnesium acetate, 0.14 μmole spermine, 0.2 ml of ribosomal suspension containing 1.42 mg of protein, and 0.3 ml of 100,000 × *g* supernatant fluid containing 0.63 mg protein. ●—●, growth curve, expt 39; ●---● specific-activity curve, expt 39; ○—○ growth curve, expt 40; ○---○ specific-activity curve, expt. 40.

carrier-free orthophosphate solution containing 1.8 × 10^5 cpm was added, and the flasks were shaken at 30° for 4 minutes. The reaction was stopped by pouring the contents of the flask into 175 ml of a partly frozen solution containing 0.2 M potassium phosphate buffer and 0.1 M sodium azide, *p*H 5.3, and all further operations were carried out at 0–1° unless otherwise specified. The cells were then washed twice with the phosphate-azide buffer and once with H_2O, and were stored at −20° until further operations could be carried out. Upon thawing, the cells were washed once again with water and were then washed repetitively with 5% perchloric acid (about 8 times) until the radioactivity in the wash fluid decreased to inappreciable levels. The time necessary for the acid washings was kept as short as possible and the temperature as close to 0° as possible. The sediment was then further washed with an ether-ethanol (2:1) solution. Hydrolysis of the RNA and isolation of the individual nucleotides were performed as described in the previous section. The ^{32}P samples were plated on slightly dished stainless steel planchets and the radioactivity was counted.

Determination of Total Cell Concentration.—Cells were counted in a hemocytometer chamber. In the early stages of the growth cycle, it was necessary to concentrate the cells for counting and this was done by sedimentation and resuspension with the aid of a "Vortex"-type mixer in a smaller volume of medium. Clumping was not a serious problem. Appropriate dilutions were made of samples of high cell density.

Determination of Dry Weight.—After the cells were washed with water three times, they were transferred to a preweighed cup and dried for several hours under an infrared lamp, and the planchet was then reweighed.

RESULTS

Protoplast-derived Ribosomes.—Early difficulties in this and other laboratories in obtaining an active cell-free system from *S. cerevisiae* suggested the need for a method which would effectively rupture the tough cell wall of this organism without inactivating the ribosomes. A procedure involving enzymatic digestion of the cell wall and subsequent disruption of the resulting protoplasts by osmotic lysis was attempted and, although tedious, was found to lead to active ribosomal preparations (Lucas *et al.*, 1963). This method was also successfully used with *S. carlsbergensis* by Barnett *et al.* (1962), whereas Williams and Novelli (1962) first solved the problem by substituting *S. fragilis* for *S. cerevisiae*. Inasmuch as our later studies showed that a cell-disruption procedure based on grinding with alumina under special conditions gave active ribosomes more rapidly and in better yield than did the protoplast procedure, this method of cell breakage was used routinely and only the results obtained by the alumina grinding method are reported in this paper.

Properties of the Cell-free System.—The yeast-ribosomal system used in the experiments described in this report possesses many features in common with bac-

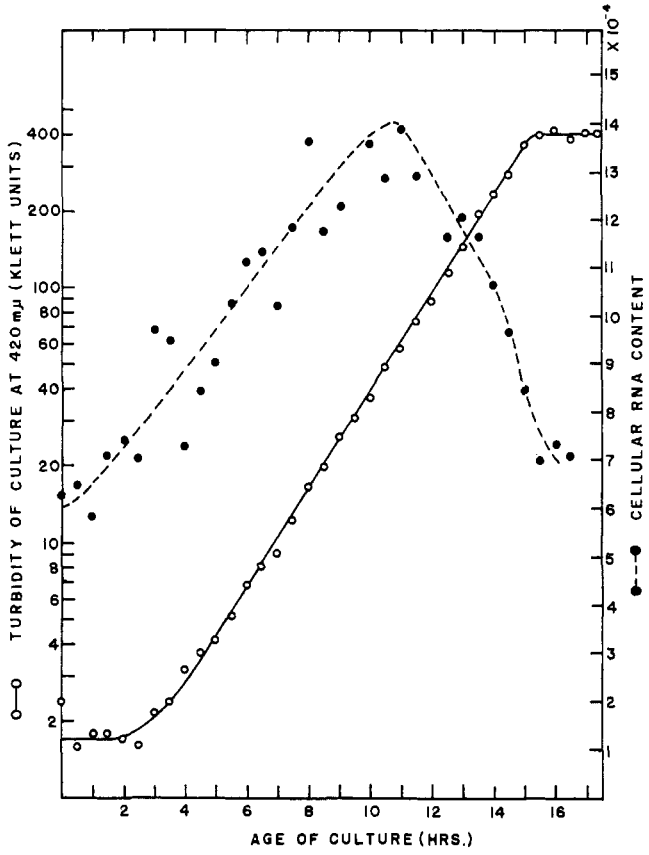

FIG. 2.—The effect of the age of the culture on the cellular content of RNA. ○—○, growth curve; ●---● cellular-RNA content, expressed as μmoles RNA-nucleotides per unit of total Klett turbidity of the sample of the yeast culture, total Klett turbidity being defined as the Klett reading at 420 mμ of a sample of the culture times the volume of the sample analyzed. The A_M for hydrolyzed RNA was taken as 10,800 (4) and corresponds very closely to the value calculated from the base ratios of total yeast RNA as given in Table II.

terial cell-free systems and with systems derived from other strains of yeast. However, it may be of value, in relation to the work to be reported here, to summarize the properties of the *S. cerevisiae* system, inasmuch as some of these properties have not yet been described in a yeast system and others differ quantitatively from those in the detailed reports of So and Davie (1963) and Bretthauer *et al.* (1963) on *S. fragilis* and *S. fragilis* X *S. dobzhanskii*, respectively.

The amino acid–incorporating system shows a strong dependence upon ATP or an ATP-generating system, is completely inhibited by RNase but not by DNase, and is also completely inhibited by puromycin but not by chloramphenicol. Similar results are obtained when the transfer step is studied, i.e., when the labeled amino acid and the *p*H 5 fraction are replaced by labeled aminoacyl-s-RNA. Under the conditions of these experiments, the time course of the reaction is linear for 10–15 minutes and is over in 30 minutes.

Both the overall reaction and the transfer step show an absolute requirement for Mg^{2+} (optimal concn 7 mM); low concentrations of spermine decrease the optimal Mg^{2+} requirement and increase the activity of the system. ATP (optimal concentration 0.8 mM) can replace the ATP-generating system with only a slight loss in activity, and a stimulation by GTP can be shown in the transfer step. The dependence on K^+ is absolute (optimal concentration 0.1 M) and no other ion tried can more than partly substitute for it. Ribosomal washings, which contain transfer enzyme, restore the lost activity of washed ribosomes as do partly purified "transfer enzymes." However, heated ribosomal washings are 50% effective and work is in progress on the identification of the heat-stable factor which does not appear to be glutathione.

Influence of Age of Culture on Activity of Cell-free System.—Figure 1 shows the ability of the yeast cell-free system to incorporate [1-^{14}C]leucine into protein plotted as a function of the age of the culture from which the system was derived. The results of two representative experiments are given. It can be seen that the incorporating activity reaches a maximum at a cell density equivalent to about 100 Klett units or about four cell doublings. From that point on the activity drops rapidly until the onset of the stationary phase and beyond. However, most of the variation in activity takes place well within the exponential phase.

Role of the Ribosome.—Any of a number of components of the cell-free system (e.g., the activity of the amino acid–activating enzymes) could effect the observed variation in amino acid–incorporating activity and we sought to distinguish between soluble components of the system and the ribosomes with respect to this responsibility. Ribosomes and 100,000 × *g* supernatant fluid were prepared from batches of cells which were harvested at various points in the log phase and a number of "heterologous" incubations were carried out in which the supernatant fluids and the ribosomes from the various batches of cells were interchanged.

It can be seen from the results presented in Table I that it makes little difference in the extent of amino acid incorporation from which batch of cells the supernatant fluid was derived. On the other hand, the activity of the system is decisively influenced by the particular batch of ribosomes used. Similar results

TABLE I

THE EFFECT OF THE AGE OF THE CULTURE ON THE ABILITY OF RIBOSOMES AND 1,000,000 × *g* SUPERNATANT FLUID TO PROMOTE [^{14}C]LEUCINE INCORPORATION INTO PROTEIN IN A CELL-FREE SYSTEM[a]

Age of Culture from Which Supernatant Fluid was Derived	Specific Activity of Protein (cpm/mg protein) Age of Culture from Which Ribosomes were Derived (hr)					
(hr)	5.5	7.5	9.5	10.5	12.0	14.0
5.5	170			333		
7.5		265			296	
9.5			573	342		167
10.5				345		
12.0		282			288	
14.0			612			149

[a] The growth curve of the culture from which the yeast cells were harvested and the composition of the reaction mixture are given in Fig. 1.

were obtained from experiments in which the various supernatant fluids were pooled (as well as *p*H 5 fraction, when this was used) and only the ribosomes were varied.[3] It is clear, therefore, that the variation in activity of the cell-free system with the age of the culture is a function of the change in activity of the ribosome.

Two points may require some emphasis here: First, in the experiments leading to the results in Figure 1 and Table I, the same amounts of ribosomal protein (and of 100,000 × *g* supernatant protein) were present in all incubation flasks. Second, the results are expressed as specific radioactivity of protein rather than total radioactivity incorporated. The results therefore reflect the *specific* ability of the ribosome to incorporate amino acids.

The localization of the effect in the ribosome rather than in the soluble fraction reduces the possibility that the apparent variation in incorporating ability could result merely from isotope dilution owing to a change in the pool sizes of amino acids or other protein precursers (e.g., aminoacyl-s-RNA). In this connection, it has recently been found that well-washed ribosomes also exhibit this effect.[3]

Variation in Cellular Content of RNA.—With the finding of a variation in ribosomal activity during the log phase, we thought it not unlikely that parallel changes might be occurring in other properties of the cell which might be related to this variation. When cells harvested at different points in the growth curve were analyzed for their total cellular RNA content, such a variation was indeed found (Figs. 2 and 3). (The results plotted in Fig. 2 were obtained using the routine method of RNA analysis and in a separate experiment [Fig. 3] were obtained by chromatographic analysis of the KOH hydrolysate for UMP.) A rise and then a decline in the cellular level of RNA takes place. Again, most of this variation occurs in the log phase.

The results of a fairly large number of experiments in which ribosomal-incorporating activity was measured indicate that the peak of activity is always close to a cell concentration corresponding to a turbidity of 100. However, the point in the growth curve at which the maximum level of RNA occurs is considerably less constant from experiment to experiment, and this variability extends to the shape of the RNA curve as well. Insufficient information is available at present to explain this variability.

[3] G. Dietz and M. V. Simpson, to be published.

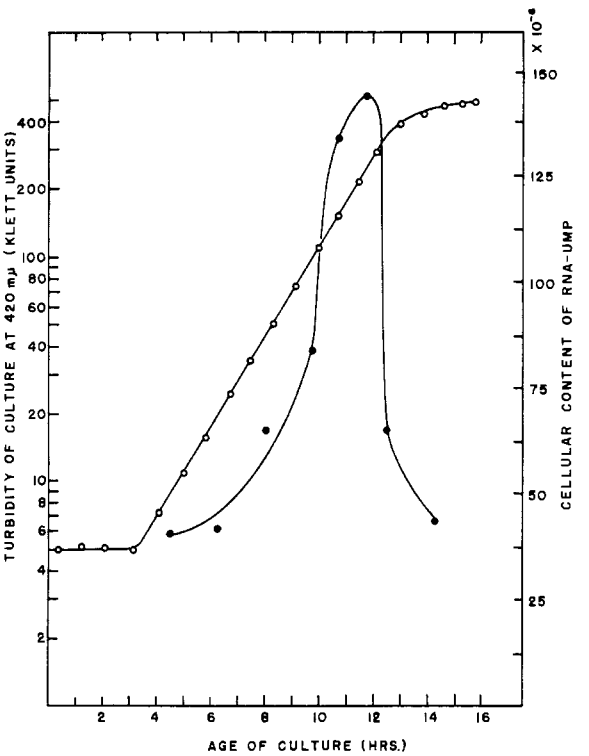

FIG. 3.—The effect of the age of the culture on the cellular content of RNA. ○—○, growth curve; ●—● μmoles RNA-UMP per unit of total Klett turbidity of the sample of the yeast culture analyzed (see legend to Fig. 2). This experiment is similar to that shown in Fig. 2 except that the RNA analysis was done by analyzing the alkaline RNA hydrolysate for UMP by paper chromatography. The values can be converted to μmoles RNA by multiplication by 3.9 (see base ratios in Table II).

Although the nature of the RNA which varies in amount with culture growth has not been determined directly, ribosomal RNA comprises so high a proportion of total cellular RNA (Wade and Morgan, 1957; Mendelsohn and Tissières, 1959) that it is likely that the observed variation in total RNA represents a variation in the number of ribosomes per cell. Experiments are in progress to verify this view directly.

The bulk of the new RNA formed does not appear to be of a different type. As can be seen from Table II

TABLE II

THE BASE COMPOSITION OF TOTAL CELLULAR RNA AS A FUNCTION OF THE AGE OF THE CULTURE[a]

Time after Inoculation (hr)	AMP (%)	CMP (%)	GMP (%)	UMP (%)	$\frac{A+U}{C+G}$
7	26.5	18.1	30.0	25.4	1.08
9.25	25.6	19.7	29.3	25.4	1.04
10.67	25.5	19.9	28.3	26.3	1.07
11.75	26.4	19.7	28.4	25.5	1.08
13.17	25.3	18.7	31.2	24.8	1.00
15.5	25.9	19.3	29.0	25.8	1.07
23.75	25.0	17.9	29.8	27.3	1.09
25.25	26.3	18.8	28.6	26.3	1.11
RNA[b]	25	20	28	27	1.08
DNA[c]	31.3	17.1	18.7	32.9[d]	1.80

[a] The growth curve in this experiment is very similar to that given in Fig. 3. The log phase extends from 3 hours to about 13.5 hours after inoculation. [b] Crestfield *et al.* (1955). [c] Chargaff (1955). [d] dTMP.

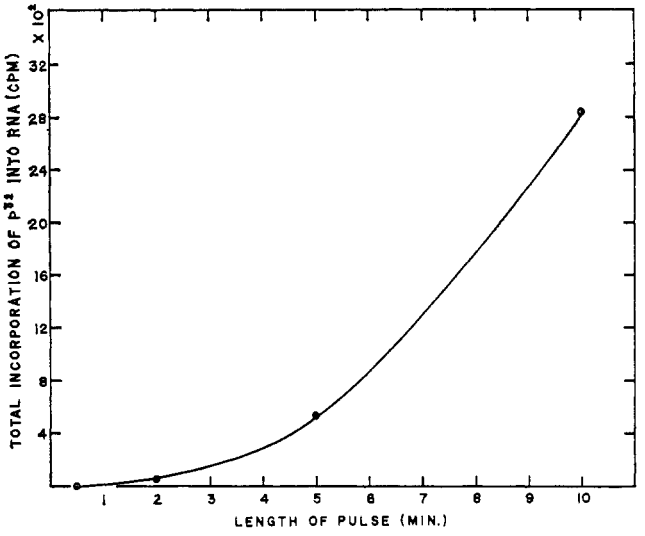

FIG. 4.—The effect of the length of the ^{32}P pulse on the incorporation of [^{32}P]orthophosphate into RNA. The cells used were obtained from a log-phase culture whose turbidity was 120 Klett units. They were resuspended in 25 ml of a medium low in phosphate at a cell concentration equivalent to 100 Klett units. Each flask contained 500 μc (5×10^8 cpm on the counter used) of inorganic [^{32}P]-orthophosphate. Other conditions and additional details are as described in the experimental section including those concerned with the isolation of the nucleotides from the KOH digest. The points on the curve represent the sum of the total radioactivity in all four nucleotides. To relate these values to the amount of ^{32}P used in the pulse, a factor of 2.8 should be applied owing to radioactive decay during the experiment, and dilution by the "low-phosphate" incubation medium should also be taken into account.

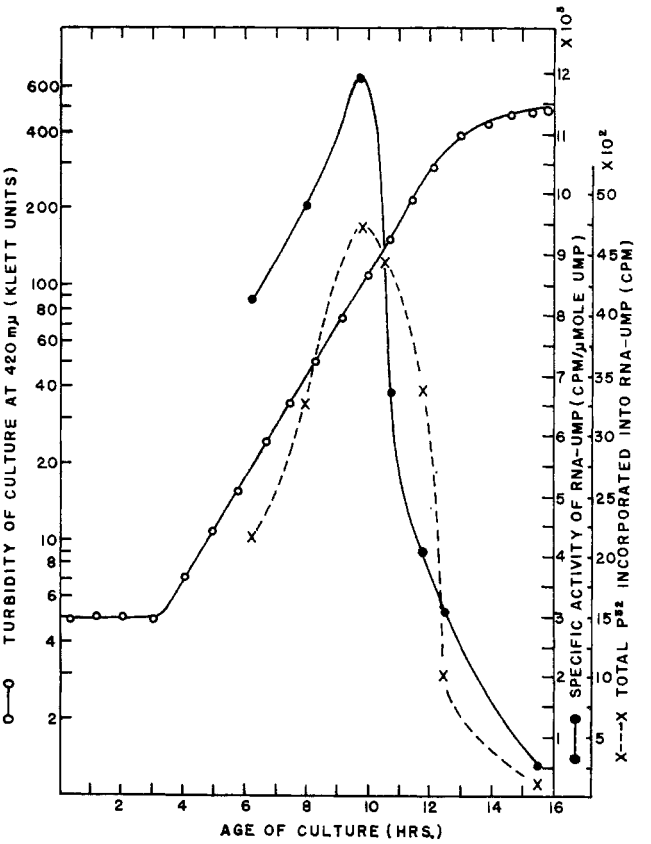

FIG. 5.—The effect of the age of the culture on its ability to incorporate a pulse of [^{32}P]orthophosphate into RNA. ●—●, total incorporation into RNA-UMP; O—O, specific activity of RNA-UMP. Each of the points represents the results obtained after a 4-minute pulse of [^{32}P]orthophosphate. Experimental details are given in the text.

its base ratios show no evidence of appreciable change during the growth period of the cells. It cannot be inferred from this, however, that small amounts of a different species of RNA (e.g., messenger RNA) are not also being formed during this period; such formation might well be masked by the large increase in ribosomal RNA. The results in the next section suggest that this may well be the case.

Rate of Synthesis of Messenger RNA.—The rise or fall observed in the number of ribosomes per cell could affect the capacity of the cell to synthesize protein, but does not provide an explanation for the variation in the *specific* amino acid–incorporating activity of the ribosome. One possible explanation for this phenomenon concerns possible differences in the availability to the ribosome of messenger RNA. Thus, a change in the rate of synthesis of this type of RNA relative to that of the ribosomes could result in an altered ratio of these substances in the cell and therefore in an altered ratio of active to inactive ribosomes.

To measure the rate of synthesis of messenger RNA during the growth cycle, cells were harvested at appropriate time intervals, and were pulsed with ^{32}P-labeled orthophosphate. To ascertain the minimal length of pulse which could be used, prior exploratory experiments were performed. The results (Fig. 4) indicated that an appreciable lag in ^{32}P incorporation occurred after the addition of the labeled phosphate. It was evident that the use of pulse times much shorter than 4 minutes would result in little radioactive phosphate being incorporated, and therefore in considerable lack of precision in the determination of the base ratios of the "pulse RNA" unless very large amounts of labeled phosphate were used. For studies on messenger RNA synthesis in *E. coli* (Gros *et al.*, 1961; Hayashi and Spiegelman, 1961), a pulse time of 4 minutes would appear to be rather lengthy and would likely result in the masking of its synthesis by the synthesis of ribosomal RNA. However, in yeast the lag which occurs, while necessitating longer nominal pulse times, also effectively shortens the actual pulse time. Moreover, Ycas and Vincent (1960), under conditions similar to those used here, were able to detect messenger RNA synthesis in yeast using pulse times severalfold longer than 4 minutes.

The results of the 4-minute pulse experiment, in which the incorporation of [^{32}P]orthophosphate into the UMP residue of RNA was measured as a function of the age of the culture, are given in Figure 5. It can be seen that the variation in both the total incorporation and the specific activity closely resemble the changes observed in ribosomal activity (Fig. 1); the maximal rate of ^{32}P incorporation occurs at a cell density equivalent to 100 Klett units, as does the maximal amino acid–incorporating activity of the ribosomes.

The extent of ^{32}P incorporation in this experiment is a measure of the rate of synthesis of new RNA; the nucleotide composition of this RNA, discussed in the following section, suggests that it may be messenger RNA. The change in the specific activity of RNA seen in Figure 5, for example its rise in the early half of the log phase, is thus in accord with the hypothesis that although the net amount of ribosomal RNA is increasing, messenger RNA is being synthesized at a rate which exceeds that of ribosomal RNA. This could result in an increase in the ratio of messenger RNA to ribosomes and could explain their increased activity in the early part of the log phase.

It should be mentioned at this point that the values for ^{32}P incorporation obtained in this experiment re-

TABLE III
APPARENT NUCLEOTIDE COMPOSITION OF ^{32}P-LABELED RNA[a]

Nucleic Acid Analyzed	2′,3′-Nucleotide	Total ^{32}P Incorporated (cpm)	Composition (%)	A + U / G + C	Purines / Pyrimidines
Total RNA	AMP	138	33.7		
5′ Pulse	CMP	78	19.1	1.73	1.05
(isotopic	GMP	72	17.6		
analysis)	UMP	121	29.6		
Total RNA	AMP	523	32.3		
10′ Pulse	CMP	329	20.3	1.58	1.03
(isotopic	GMP	300	18.5		
analysis)	UMP	468	28.9		
Total yeast	AMP		25.8		
RNA	CMP		19.0	1.08	1.13
(chromato-	GMP		29.3		
graphic	UMP		25.8		
anal.)					
Yeast s-RNA[b]	AMP		16.1		
	CMP		31.4	0.52	0.99
	GMP		30.7		
	UMP		15.9		
Yeast DNA[c]	AMP		31.7		
	CMP		17.4	1.80	1.00
	GMP		18.3		
	TMP		32.6		

[a] The values in the row labeled "Total Yeast RNA" are the mean of the values given in Table II. These and the figures taken from the literature in the following two rows were determined by chemical (as opposed to isotopic) analyses after chromatographic isolation. In the case of s-RNA, the "minor" nucleotides, totaling about 6% of the total nucleotide content, are not included in the values given, which total 94%. [b] Nihei and Cantoni (1964). [c] Chargaff (1955).

flect only the *rate* of synthesis of the presumed messenger RNA; they say nothing about the *amount* present. If the change in the rate of ^{32}P incorporation merely reflected a change in the turnover rate of messenger RNA, the net amount would not change. Further, the assumption has been made in the interpretation of these results that the apparent change in the rate of [^{32}P]phosphate incorporation with the age of the culture does not result from changes in the pool sizes of orthophosphate or of other phosphate-containing precursors of RNA. Preliminary testing of this assumption indicates that total acid-soluble 260 mμ-absorbing material remains constant throughout the log phase.

Base Composition of Pulse-labeled RNA.—A great deal of evidence has accumulated (Simpson, 1962; Stevens, 1963) that it is possible to reveal the presence of messenger RNA by its relatively high turnover rate in comparison with other types of RNA (e.g., s-RNA or ribosomal RNA) and by the determination by isotopic analysis of its base composition which also differs from that of ribosomal or s-RNA. The base composition of this RNA frequently corresponds to that of homologous DNA or, in the case of T_2 phage, to the phage DNA. The results in Table III show that the base composition of the ^{32}P-labeled RNA is neither that of total cellular RNA (the bulk of which is ribosomal RNA), nor that of s-RNA. It is of additional interest that the shorter pulse time gives values for the base composition which more closely resemble those of yeast DNA.

It might be pointed out, however, that if the recent reports from a number of laboratories (Guild and Robison, 1963; Hayashi *et al.*, 1963; Tocchini *et al.*, 1963), in which only a single strand of DNA has been shown to be active as a template for messenger RNA, apply generally under all conditions, then the composition of messenger RNA need not reflect the composition of homologous DNA (although it is possible for it to do so, given certain base compositions of the DNA). Under these circumstances, other experimental approaches will be necessary to establish whether or not the rapidly synthesized RNA in this experiment is in fact messenger RNA. It might be added, at this point, that messenger RNA is being used in this report as an operational term, without regard to whether or not the material being measured actually possesses the function of a "messenger."

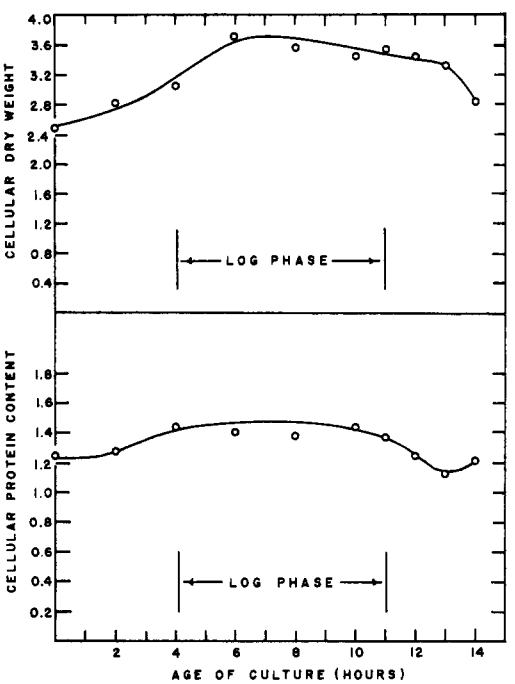

FIG. 6.—Changes in the dry weight and the protein content of the yeast cell with the age of the culture. The ordinate values are expressed in μg per unit of total Klett turbidity (see legend to Fig. 2).

Changes in Protein and Dry Weight.—It can be seen from Figure 6 that the dry weight per cell changes appreciably during the log phase. However, while the

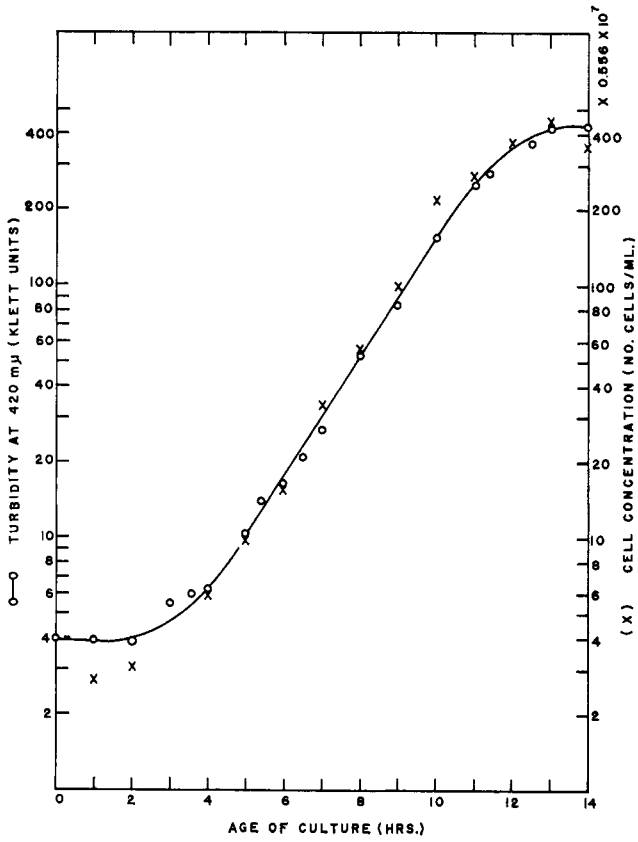

FIG. 7.—Comparison of the turbidity (in Klett units) of the culture with the cell count.

variations in protein content tend to follow those of the dry weight, they are much smaller.

Cell Count.—In order to be certain that the absorbancy measurements were actually a measure of the true rate of cell multiplication, total cell counts were performed on the growing culture. Figure 7 shows both absorbancy and cell count plotted linearly as a function of the time of growth. For purposes of comparison of the two curves, the values for the cell count were multiplied by a factor (0.556). It can be seen that the two curves are virtually superimposable. In other experiments, large differences between the absorbancy and cell-density curves begin to appear later in the stationary phase.

DISCUSSION

The results reported here indicate that during the log phase of the growth cycle of *S. cerevisiae*, certain fundamental properties of the cell undergo change. These properties include the number of ribosomes per cell, the ability of the cell to synthesize ^{32}P-pulse-labeled RNA (presumably messenger RNA), and the capacity of the ribosome to incorporate labeled amino acids into protein in a cell-free system.

Variation in the cellular RNA content in microorganisms is well known. Comparisons of the RNA content in the lag, log, and stationary phases of the growth cycle (Belozersky, 1959; Dean and Hinshelwood, 1959; Neidhardt and Magasanik, 1960; Cohen, 1948; Malmgren and Heden, 1947) have shown that a rapid accumulation of RNA occurs in the lag phase and that this increase in RNA content is a reflection of the synthesis of new ribosomes (Wade and Morgan, 1957; Mendelsohn and Tissières, 1959). The RNA content is also known to vary directly with the rate of growth as has been demonstrated in balanced growth experiments under conditions of different growth rates (Neidhardt and Magasanik, 1960; Schaechter *et al.*, 1958). The increased RNA content which occurs in the lag phase does not remain at a constant level in the exponential phase. Depending upon the microorganism studied and presumably upon the conditions of growth, a continuous decline is observed after the initiation of the log phase (Belozersky, 1959; Dean and Hinshelwood, 1959; Malmgren and Heden, 1947; Morse and Carter, 1949; Buetow and Levedahl, 1962) or, as we observed with yeast, a rise during several log-phase generations and then a rapid decline (Wellerson and Tetrault, 1955; Doerfler *et al.*, 1962).

When cell-free systems of *E. coli* (strain K-12) were tested[3] for amino acid–incorporating activity as a function of the age of the culture, the results were strikingly similar to those obtained with yeast. These results with *E. coli* are in accord with those of Doerfler *et al.* (1962) who, in a recent report published after this work was completed, showed that the activity of an *E. coli* cell-free amino acid–incorporating system varied during the log phase. Earlier, Lamborg and Zamecnik (1960) cautioned that for maximal amino acid incorporation *E. coli* cells must be harvested in the earliest stage of log-phase growth.

The combination of an increase in activity of the ribosome and an increased number of ribosomes leads to a greatly increased capacity of the cell for protein synthesis. Yet analysis of the cellular protein content during the period in the log phase in which this increased capacity develops showed little or no increase in protein level. Evidently, the cell is not making use of this increased synthetic capacity. The apparent lack of a function for this added capacity for protein synthesis suggests that it may be the result of inefficient control mechanisms—an "overshoot" in the intense synthetic activity occurring in the lag phase. The subsequent decreases observed in the various parameters measured might then be the result of the cellular control mechanisms' "catching up." Alternatively, the increased capacity for protein synthesis may be the reflection of an increased rate of protein turnover—with no observable net synthesis. The necessity for the increased turnover could result from the necessity for destroying an excess of certain enzymes which were needed in the lag phase and synthesizing others. A third possibility is that the phenomenon, either in part or in its entirety, may result from gradual changes in the composition of the medium. Recent preliminary experiments,[3] in which ribosomal activity at constant cell density was followed as a function of time, lend support to the view that the decline in ribosomal activity observed in the latter half of the log phase is indeed related to medium composition.

It is of interest in this connection that Ephrussi *et al.* (1956) have concluded that the reciprocal changes which take place during the log phase of growth of *S. cerevisiae* in the capacities of the cells for respiration and for aerobic fermentation result from changes in the glucose concentration of the medium. Whether or not these changes occur under the conditions used here and whether they influence ribosome synthesis and activity are subjects for future investigation.

In terms of the chemical mechanisms of protein synthesis, a question that might be posed is that of which ribosomal constituents are responsible for the variation in activity of the ribosomes. It is clear that the nucleotide composition of the ribosomal RNA does not change throughout the log phase nor could appreciable variation in the RNA-protein ratio of the ribosomes be found.[3] The change in the rate of synthesis of mes-

senger RNA suggests that the messenger-RNA content of the ribosome may differ in different parts of the log phase. Such a condition could also lead to a variation of the polysome content of the cell, and these questions are being investigated. Differences in the transfer enzyme content of the ribosomes do not appear to be the explanation because 100,000 × *g* supernatant fluid, which contains these enzymes, was used in the incubation mixture.

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Cyclopropane Fatty Acid Synthetase: Partial Purification and Properties*

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The cyclopropane fatty acid synthetase from *Clostridium butyricum* has been partially purified. The enzyme system transfers the methyl group of *S*-adenosylmethionine to a monounsaturated fatty acid chain of phosphatidylethanolamine to form a cyclopropane–fatty acid chain. The enzyme system is stimulated by anionic surfactants and inhibited by cationic and neutral surfactants. The Michaelis constants for phosphatidylethanolamine and *S*-adenosylmethionine are 5.3×10^{-4} M and 5.7×10^{-5} M, respectively. The Arrhenius plot for the enzyme reaction is discontinuous over the temperature range 0–40°. The heat of activation for the enzyme reaction is lowered by the addition of the anionic surfactant sodium lauryl sulfate.

The occurrence in lactobacilli of a 19-carbon fatty acid containing a cyclopropane ring was first demonstrated by Hofmann and co-workers (Hofmann *et al.*, 1952), who characterized the compound as *cis*-11,12-methyleneoctadecanoic acid (Hoffmann *et al.*, 1952). A 17-carbon cyclopropane fatty acid from *Escherichia coli* was described by Dauchy and Asselineau (1960) and characterized as *cis*-9,10-methylenehexadecanoic acid by Kaneshiro and Marr (1961). The methylene carbon of lactobacillic acid has been shown to be derived from the methyl group of methionine (Liu and Hofmann, 1962), and several workers have demonstrated the incorporation of methionine methyl groups into cyclopropane fatty acids (O'Leary, 1959, 1962; Chalk and Kodicek, 1961; Law *et al.*, 1963). Zalkin and Law (1962) and Zalkin *et al.* (1963) have recently demon-

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