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*STUDIES ON THE KINETICS OF PROTEIN SYNTHESIS IN YEAST**

BY R. J. YOUNG,† H. K. KIHARA, AND H. O. HALVORSON

DEPARTMENT OF BACTERIOLOGY, UNIVERSITY OF WISCONSIN

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It is now generally agreed that ribosomal particles are the primary site of protein synthesis. In yeast, this evidence is supported by studies on the incorporation of amino acids *in vivo*[1, 2] and *in vitro*[3] and by the finding on ribosomes of bound protein[4] and enzymes[5] which are presumably newly synthesized protein. One question raised by these findings is what percentage of the ribosomal protein is represented by newly synthesized polypeptides and what is the relationship between this material and the structural proteins of the ribosome? In an attempt to further characterize the nascent ribosomal protein, this material was isolated and compared with the structural protein of the ribosomes. These two classes of protein differed not only in their composition and properties but also in the kinetics of synthesis. The nascent protein has a transient existence on the ribosome and the kinetics of its turnover is sufficient to account for the over-all rate of protein synthesis.

Methods.—A diploid yeast (*Saccharomyces dobzanskii* × *Saccharomyces fragilis*) was used in these experiments. Unless otherwise specified, all cultures were grown in a sulfur-low, synthetic medium prepared by adding to one liter of water: NH_4Cl, 6.4 gm; K_2HPO_4, 17.4 gm; succinic acid, 11.6 gm; $CaCl_2$, 0.2 gm; $MgCl_2$, 0.2 gm; $(NH_4)_2SO_4$, 5 mg; sulfur-free trace elements; and vitamin mixture. The cultures were grown at 30° in shaken Erlenmeyer flasks. Under the conditions employed, the generation time was about 190 min.

For the kinetic studies on S^{35} and C^{14} amino acid incorporation, yeast cells were grown in the above medium until the optical density had reached a level of about

0.20–0.30 as measured in a Beckman spectrophotometer at 600 mμ in a 10 mm cell. The sulfur content of the medium is sufficient to provide exponential growth to optical density levels above 1. For the S^{35} experiments, carrier-free $H_2S^{35}O_4$ was added at a level of 7.5 mC per 100 ml of culture. At intervals, samples were withdrawn immediately into tubes containing 10% TCA or poured into beakers containing crushed, frozen medium.[6] In both cases, further incorporation was immediately arrested. The samples were then fractionated as described in the text. Ribosomes were prepared as previously described.[5]

Protein was measured by the method of Lowry *et al.*[7] using crystalline serum albumin as the standard. For radioactivity experiments, aliquots of the various fractions were evaporated to dryness on stainless steel planchets and counted in a gas flow counter, and their radioactivities were corrected to infinite thinness.

Materials.—Cacodylic acid was obtained from the Fisher Scientific Company. Diisopropylfluorophosphate was obtained from Dr. R. M. Bock and the amino acids from the California Corporation for Biochemical Research. Deionized glass-distilled water was used for all experiments. Carrier-free $H_2S^{35}O_4$ was obtained from the Oak Ridge National Laboratory. Totally labeled amino acids were prepared by growing *Hydrogenomonas facilis* on $C^{14}O_2$ (52.6 per cent isotope excess) and preparing an acid hydrolysate of the proteins as described by Roberts *et al.*[8]

Results.—Kinetics of $S^{35}O_4^=$ incorporation: As a preliminary step to the study of ribosomal incorporation, an investigation was undertaken on the kinetics of incorporation of $S^{35}O_4^=$ into exponentially growing yeast. At intervals, 2.0 ml aliquots were removed by means of a hypodermic syringe and squirted into tubes containing 5 ml of crushed, frozen medium. The cells were then washed with ice water and precipitated and washed with 10 per cent TCA containing 1 *M* $(NH_4)_2SO_4$. The pellet was resuspended and an aliquot extracted several times with hot TCA. The extracted pellet was solubilized with 5 *N* NH_4OH and counted as a measure of the incorporation into the protein fraction. The results are shown in Figure 1. As can be seen, the total uptake of radioactivity has not reached a steady state by 600 sec. The cold TCA-soluble fraction was treated with Ba^{++} to remove the sulfate; the remaining radioactivity was found almost exclusively in S-containing amino acids. The rate of incorporation into this fraction is much more rapid than that of the growth rate and follows kinetics expected of that of a precursor material.[8] The initial rate of incorporation into proteins is only about 20–25 per cent of that of the labeling of the cold TCA-soluble pool. Of interest was the finding that the rate of incorporation into the hot TCA-soluble fraction, which includes the soluble RNA complexes, was slower than that of the rate of labeling of the protein fraction.

The rate of incorporation of $S^{35}O_4^{--}$ into the hot TCA-insoluble fraction was followed at shorter intervals by pipetting aliquots directly into tubes containing hot TCA and nonradioactive substrate. As is shown in Figure 2, the rate of incorporation into this fraction is linear over the initial 90 sec of incubation. The rate of incorporation extrapolates back to the addition of the isotope, and thus the delay in incorporation is less than 5 sec and is probably on the order of 1–2 sec.

Incorporation of C^{14} amino acids into ribosomes and soluble proteins: The kinetics of incorporation of totally labeled C^{14} amino acids into ribosomal and soluble proteins of exponentially growing yeast were studied by the following experiments.

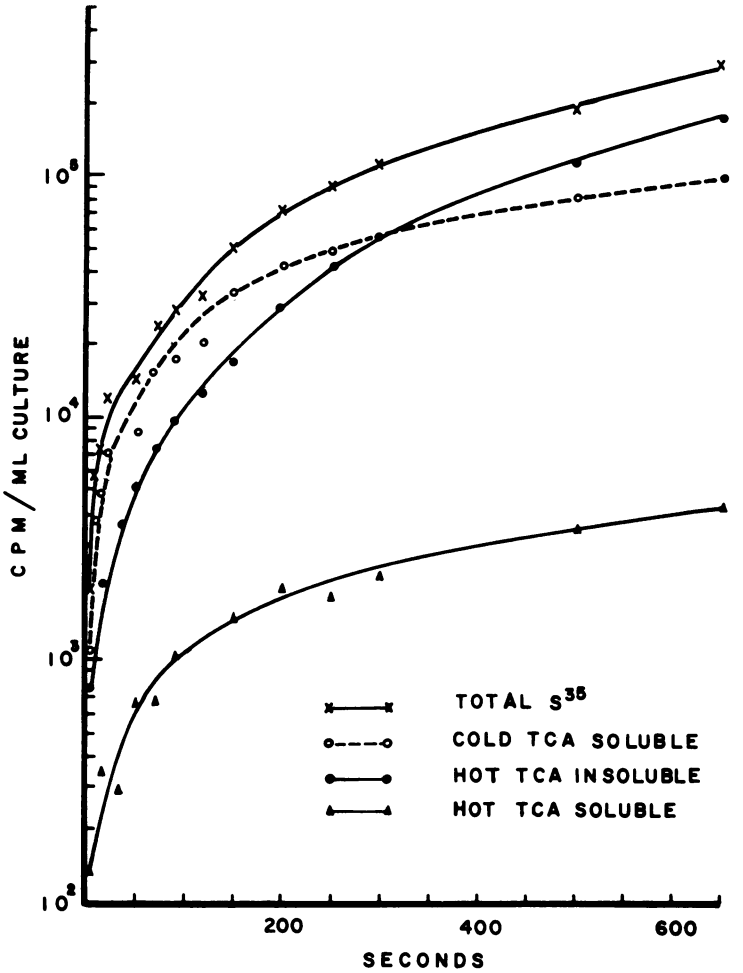

FIG. 1.—Kinetics of $S^{35}O_4^{=}$ incorporation into growing yeast. At the indicated times, aliquots were removed, washed, and fractionated as indicated in the text.

At intervals after addition of the isotope, samples were poured on ice chilled to $-20°$. The cells were collected by centrifugation, washed twice with cold water, and resuspended in cold 6×10^{-4} *M* Mg cacodylate buffer, pH 7.2. The cells were broken in a French press and the ribosomal and supernatant fractions collected as described elsewhere.[5, 6] The soluble protein fraction was precipitated from the supernatant with hot TCA; the precipitate was washed with an aqueous solution of unlabeled amino acids and finally solubilized with 5 *N* NH_4OH. The results are shown in Figure 3. The rate of incorporation into the ribosome fraction is about 4 × that of the soluble proteins. At 80 sec, excess unlabeled amino acids were added. Within 10 sec, all incorporation into the ribosome fractions was terminated and the specific activity of the ribosome dropped to a constant level after 120 sec. This decrease is paralleled by a net increase in the soluble protein

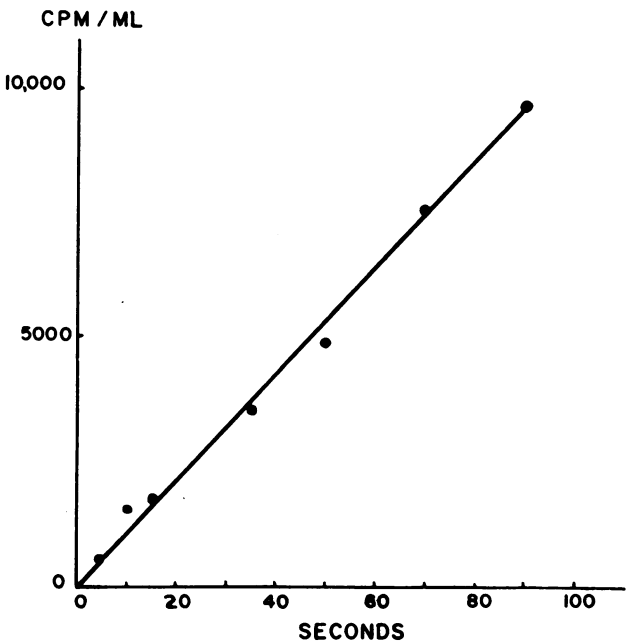

FIG. 2.—Time course of $S^{35}O_4^-$ incorporation into yeast proteins. $S^{35}O_4^-$ was added to a growing culture of yeast. At intervals, 2 ml aliquots were removed and pipetted into tubes containing boiling 10 per cent TCA and unlabeled sulfate, methionine, and cysteine. The pellets were washed twice with 10 per cent TCA, solubilized with 5 *N* NH_4OH and counted for radioactivity.

fraction. From the pool size and kinetics of decrease, approximately 10 sec or less of synthesis is associated with the ribosome fraction.

The nondisplaceable fraction presumably represents the newly synthesized structural protein of the ribosome. As is described by Yin and Bock[9] and shown below, about 95 per cent of the protein of ribosomes is insoluble at neutral pH and apparently serves as a structural protein. The kinetics of structural protein synthesis can thus be measured by following the formation of polypeptides which are not displaced from ribosomes by treatment for 120 sec with unlabeled amino acids. As indicated by the center curve of Figure 3, approximately 50 per cent of the radioactivity is associated with this nondisplaceable fraction.

Fractionation of yeast ribosomes: The use of C^{14}-labeled amino acids to study the turnover rate of protein bound to ribosomes is difficult because of delays in its exchange with the large pool of free amino acids in yeast. This problem is less serious with $S^{35}O_4^{--}$, as the pool of precursor S in yeast can be specifically reduced by growing in a medium with a reduced concentration of sulfur. Preliminary to the experiments on incorporation of $S^{35}O_4^{--}$, the distribution of S in ribosomes was analyzed.

Yeast cells were grown in a synthetic medium containing $S^{35}O_4^{--}$ (1.628×10^6 cpm/mg S). The cells were washed several times, resuspended in cacodylic buffer, and broken in French press. After removal of the debris, the supernatant was spun for 90 min at 40,000 rpm in the angle head rotor (40) of the Spinco Model L

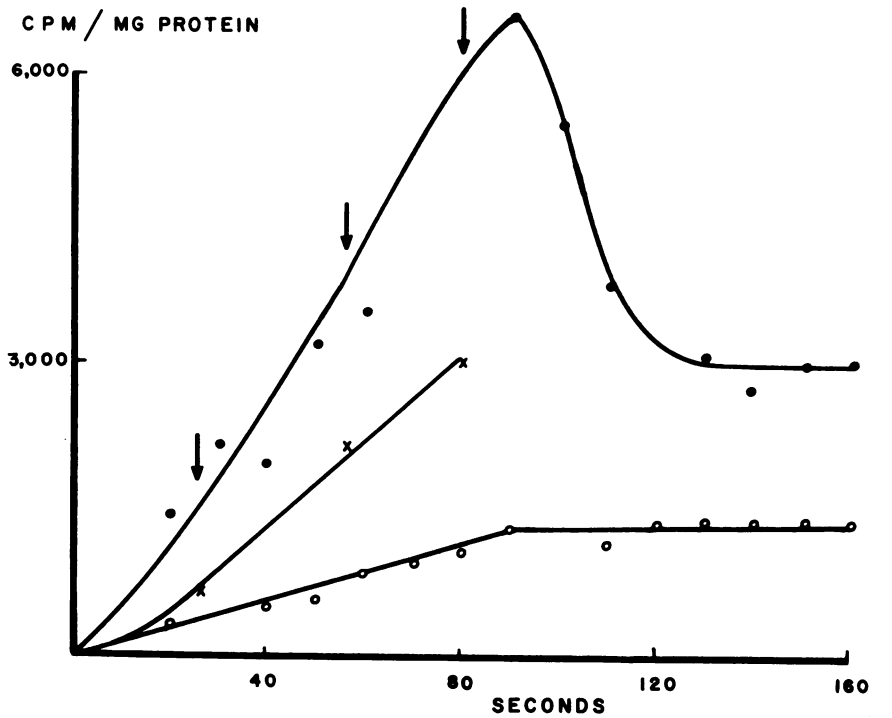

FIG. 3.—Kinetics of the incorporation of C^{14} labeled amino acids into ribosomes and soluble proteins. C^{14}-labeled *Hydrogenomonas* protein hydrolysate (4.4×10^5 cpm/ml; 54 μg. total N) was added to a growing culture of yeast (600 ml). The total free amino acid pool in this culture is about 1,500 μg. Since only a small fraction of the labeled amino acids are assimilated during the short incubation period, the free amino acid pool is not significantly altered by the added amino acids. At intervals, 40 ml aliquots were removed and treated as described in the text. At 30 and 60 seconds, 40 ml aliquots were transferred to separate flasks containing unlabeled amino acids and incubated for an additional 120 seconds. At 80 seconds, an enzymic digest of casein was added to a final concentration of 1 per cent. The specific activity of C^{14} in supernatant protein (O), total ribosomes (●), and nondisplaceable fraction of ribosomes (×) were determined as described in the text.

centrifuge. The supernatant was retained for further analysis. The ribosome fraction was repeatedly layered (3 times) on a sucrose density gradient (5–20%) and centrifuged in the swinging bucket rotor (SW 25) for 4 hr at 23,000 rpm until a constant specific activity was obtained.[5] An aliquot of the purified ribosomes was degraded with 30 μg/ml ribonuclease in 1 M urea.[9] To prevent possible protease action during digestion, 1 μmole/ml of diisopropylfluorophosphate was added before the addition of ribonuclease. The reaction mixture was allowed to stand for 1 hr. at 37° and then kept overnight at 4° to allow complete precipitation of the structural protein. The precipitate was washed three times with water, dissolved in 8 M urea and 1 per cent Dupanol, and reprecipitated by dialysis against water. The final protein was again resuspended in urea and Dupanol. The soluble protein of the cell as well as the protein solubilized from the ribosomes was precipitated with hot TCA, dissolved in 5 N NH_4OH, reprecipitated with TCA, and finally washed with alcohol and ether before assay.

Table 1 summarizes the distribution of S and protein in yeast ribosomes. The ribosome protein is primarily (96%) of the "structural type." A minor component is soluble (4%) and contains enzymes[5] as well as the same classes of proteins[4] found normally in the soluble supernatant. The sulfur content of the ribosomal soluble protein is similar to that of the protein of the supernatant fraction, whereas the

TABLE 1

COMPOSITION OF S^{35}-LABELED YEAST RIBOSOMES

Ribosomes	Sulfur (% wt.)	Protein in fraction (%)
Total	0.32	100
Structural	0.30	96
Soluble	0.53	4
Supernatant Protein	0.63	..

structural protein is low in S. These results are in agreement with the finding that the ribosomal soluble proteins are nascent precursors of the supernatant proteins.[4] During the ribonuclease digestion of the ribosomes, low molecular weight peptides are released, some of which are associated with nucleotides.[10] An examination of this fraction is under current investigation.

Incorporation of $S^{35}O_4^{--}$ into ribosomes and soluble proteins: Measurement of the incorporation of $S^{35}O_4^{--}$ appeared to be the method of choice for studying protein synthesis in yeast. The kinetics of S^{35} incorporation into ribosomes and soluble proteins were investigated in a manner analogous to that described in Figure 3 for work with C^{14}-labeled amino acids. The results are shown in Figure 4. The ribosome fraction was labeled at a rate, which in parallel experiments was 6 times faster than that of the soluble proteins. Upon addition of methionine, cysteine, and unlabeled sulfate, the specific radioactivity of the ribosome fraction drops 85 per cent within 10 sec. These findings suggest that the majority of the acid-stable S of ribosomes formed in a short period from $S^{35}O_4^{--}$ is associated with nascent rather than structural protein.

Addition of unlabeled S to S^{35}-labeled yeast cells: To further characterize the size of the nascent protein fraction in yeast ribosomes, 0.1 *M* $(NH_4)_2SO_4$, cysteine, and methionine were added to a suspension of S^{35}-labeled yeast cells. At 0, 40, and 160 sec, samples were withdrawn, and soluble protein and ribosomes were collected as previously described. The specific activities of the two fractions recorded in Table 2 show no detectable decrease, whereas following a brief exposure to isotope (Fig. 4), most of the incorporated S^{35} is displaced by unlabeled precursor. These findings further support the conclusion that the nascent protein represent only a small fraction of the total protein of yeast ribosomes.

Discussion.—In yeast ribosomes, Yin and Bock[9] isolated over 90 per cent of the ribosomal protein as strongly aggregating proteins with a predominant molecular

TABLE 2

THE EFFECT OF THE ADDITION OF UNLABELED SO_4^{--} CYSTEINE, AND METHIOHINE ON THE S^{35} CONTENT OF FRACTIONS FROM S^{35}-LABELED YEAST CELLS

Period of displacement (sec)	Protein (cpm/mg protein)	Ribosome (cpm/E_{260}/ml)
0	1.025×10^7	2.24×10^5
40	1.045×10^7	2.37×10^5
160	1.02×10^7	2.39×10^5

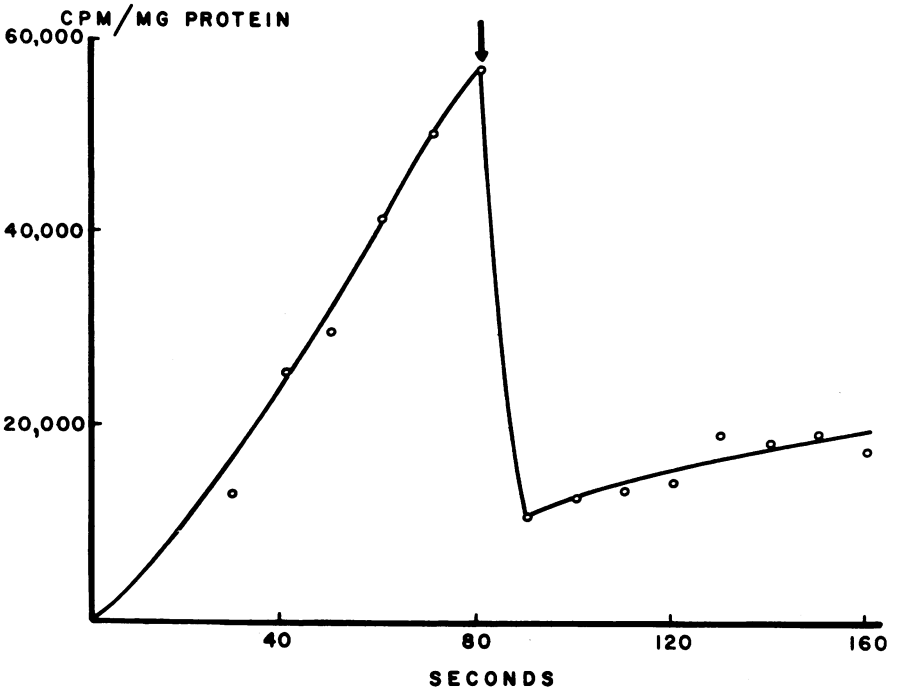

FIG. 4.—Incorporation of $S^{35}O_4^-$ into ribosomes of yeast. Experimental conditions were similar to those of Figure 3 except that $S^{35}O_4^-$ was used. At 80 seconds, unlabeled $(NH_4)_2SO_4$, cysteine, and methionine were added to a final concentration of 0.1 *M* each.

weight of 12,000. These polypeptide chains were low in amino acids containing S and had similar solubility and electrophoretic properties but at least 8 different N-terminal amino acids. These polypeptides aggregate to form complexes of 50,000 molecular weight; 40 such protein units are found per ribosomal particle.

In addition to the insoluble structural protein, a minor fraction of the ribosomal protein can be isolated as soluble heterogeneous protein. The classes of S^{35}-labeled proteins released from ribosomes are identical to the classes of soluble protein present in the cytoplasm.[4] In addition, a number of enzymes including RNAase,[11] β-glucosidase,[5] and triose-phosphate dehydrogenase,[12] have been solubilized from these particles. As described in the present paper, this fraction contains about four per cent of the ribosomal protein and has a S content which is about twice that of the structural protein.

The kinetic studies on incorporation of C^{14} amino acid and S^{35} as SO_4^{--}, support the findings in *E. coli*[6] that the soluble fraction from ribosomes contains the nascent proteins which are precursors of the soluble proteins. Evidence supporting a ribosomal-bound β-glucosidase has already been presented.[5] The ribosomal-soluble fraction represents a molecular weight of about 89,000 per ribosome. The size of the nascent polypeptide chain is probably a fraction of this, since some enzymes such as RNAase[11] are associated only with ribosomes and others such as β-glucosidase have high molecular weights. In addition, peptides have been identified in yeast ribosomes.[10] Some authors[6, 13] have described nascent protein as growing polypeptide chains of about 10,000–20,000 molecular weight.

The turnover rate of nascent protein can be calculated from the following data. The generation time of this diploid yeast in synthetic medium is about 190 min, which is equivalent to a growth rate of 0.0061 per cent increase in mass/sec. The protein content is 47 per cent of the dry weight, which is equivalent to about 3.3×10^7 molecules of protein per cell (10^5 average molecular weight). The number of ribosomes per cell is 5.5×10^5; these have a molecular weight of 4.5×10^6 and are 52 per cent protein.[5] Thus, the average yeast cell each second will increase its number of ribosomes by 33.5 and its polypeptides by 20,100, assuming the polypeptides have an average molecular weight of 10,000. If all the ribosomal particles were active in nascent protein synthesis, then the average time required to synthesize a polypeptide chain would be about 27 sec.

Assuming that completed nascent proteins are rapidly displaced from the ribosomal particles, then only the molecules being synthesized during an average period of 27 sec remain attached. These calculations agree approximately with the kinetics of displacement of isotope from prelabeled ribosomes. A 10-sec exposure to unlabeled $(NH_4)_2SO_4$, cysteine, and methionine was sufficient to displace S^{35} from ribosomes recovered from yeast treated with $S^{35}O_4^{--}$ for 80 sec, while about 40 sec were required to displace C^{14}-labeled amino acids. The delay in the latter may be due to the fact that the precursor S pools are low and reduced under growth in medium low in S; the free amino acid pool of the remaining amino acids are high, and a longer time is required to influence their specific activity.

The rate of synthesis of ribosomal structural protein can also be calculated. Each second, the average yeast cell must synthesize 5,360 polypeptides (average molecular weight of 12,000) to account for new ribosome formation. The rate of amino acid incorporation into structural polypeptide, therefore, is about $^1/_3$ of that for soluble protein synthesis. Thus, in 90 sec a quantity of ribosomal structural protein is formed equal to the steady-state concentration of nascent protein. This is confirmed in the data of Figure 3, when after 80 sec of labeling with C^{14} amino acids half of the radioactivity was associated with the structural protein. The data of Table 2 indicate that added unlabeled SO_4^{--}, cysteine, and methionine do not displace S^{35} from labeled cells in the steady state. These results provide an explanation for the experiments of Osawa and Hotta,[1] who failed to detect any displacement of C^{14} amino acids from the ribosomal fraction of yeast after 18 min of previous exposure to labeled amino acids. After 18 min, the nascent protein represents only a few per cent of the newly formed ribosomal protein.

Summary.—The kinetics of incorporation of $S^{35}O_4^{--}$ and C^{14} amino acids have been followed in exponentially growing cultures of yeast. Within a few seconds, the isotopes are incorporated into protein, appearing first in the ribosomal fraction as nascent protein. After 80 sec exposure of growing yeast cells to C^{14}-labeled amino acids or $S^{35}O_4^{--}$, the addition of unlabeled amino acids or unlabeled cysteine, methionine, and SO_4^{--} caused a rapid decrease in the concentration of C^{14} or S^{35}, respectively, held by the ribosomes. This response indicated that nascent protein synthesized on the ribosomes is displaced quickly upon completion of its synthesis into the soluble protein pool. Nascent protein represents only a small fraction of the ribosomal protein; however, its turnover rate is sufficient to account for the over-all rate of protein synthesis of the cell.

The methods employed furnish an estimate of the kinetics of synthesis of the structural protein of ribosomes.

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† Present address: Enzyme Institute, University of Wisconsin, Madison, Wisconsin.

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*BIOCHEMICAL CONTROL OF STOMATAL OPENING IN LEAVES**

BY ISRAEL ZELITCH

BIOCHEMISTRY DEPARTMENT, THE CONNECTICUT AGRICULTURAL EXPERIMENT STATION

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In the course of previous studies on the role of glycolic acid in leaf metabolism, it was found that α-hydroxysulfonates are effective inhibitors of the enzyme glycolic oxidase.[1] Treatment of leaves with these substances resulted in rapid and extensive changes in the products formed during photosynthesis.[2, 3] More recently, visible physiological effects have been observed when tobacco leaves are treated under certain environmental conditions. When slightly wilted leaves were supplied with these glycolic oxidase inhibitors during high water stress, in bright sunlight, the water vapor lost by transpiration decreased with the result that such leaves regained their turgidity more rapidly than control leaves.

Most of the water transpired by leaves apparently escapes through the stomata.[4] The width of the stomatal apertures is controlled by osmotic forces in the kidney-shaped guard cells which surround these openings. When the guard cells are