STUDIES ON PROTEIN AND NUCLEIC ACID TURNOVER IN GROWING CULTURES OF YEAST*

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INTRODUCTION

The concept of the "dynamic state of body constituents", originally proposed for adult Metazoa[1] has recently been applied to various other organisms[2]. As pointed out by HOGNESS *et al.*,[3] for the "dynamic state" to be of physiological significance at the cellular level, the rate of degradation must represent a significant fraction of the rate of synthesis.

Heterogeneity and varying environmental conditions make it difficult to test the concept of a "dynamic state" in mammalian systems. Studies with growing *Escherichia coli*, in which these difficulties are obviated, have shown that the rate of protein degradation is negligibly small[3,4]. However, it is not clear how extensive the intracellular degradation is in resting microorganisms.

This problem is amenable to an experimental approach in yeast culture where homogeneous conditions can be maintained. Resting yeast cells, like Metazoa can degrade both nucleic acid (NA) and protein to a significant extent[5]. The purpose of the investigation reported here was to compare the rate of intracellular degradation of NA and protein in growing and resting yeast cultures.

MATERIALS AND METHOD

Organism used and conditions of growth

Saccharomyces cerevisiae strain LK2G12 was used in these experiments. Unless otherwise specified, all cultures were grown in a medium prepared by adding to one liter of water: $(NH_4)_2SO_4$, 4 g; K_2HPO_4, 8.7 g; succinic acid, 5.8 g; glucose, 30 g; $CaCl_2$, 0.3 g; $MgSO_4$, 0.5 g; trace elements, 1.0 ml; and vitamin mixture, 1 ml. The cultures were grown at 30°C in shaken Erlenmeyer flasks. Under the conditions employed, the generation time in the exponential phase of growth is about 90 minutes.

Measurement of growth

During the exponential phase of growth, the protein content per cell was found to be proportional to the optical density. Therefore, for reasons of convenience, growth was followed by measurements of the optical density at 420 mμ. Under these conditions 0.100 O.D. = 4.37 μg protein N/ml.

Preparation of samples

The cell suspensions were centrifuged in the Sorvall centrifuge at 15,000 × *g* for 3 min. Aliquots of the supernatant were removed and plated directly for isotope assay. The cell residue was fractionated by the SCHNEIDER method[7] into a cold trichloroacetic acid (TCA)-soluble, hot TCA-soluble and hot TCA-insoluble fractions. The purines were isolated by hydrolyzing the hot TCA extracts

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with 1 N HCl, neutralizing the hydrolyzates with NH_3 and precipitating with silver[8]. Free purines were liberated by extracting the precipitates with hot HCl and repeatedly evaporating the extracts to dryness to remove the excess HCl. In certain cases the purity of the purines was ascertained by ion-exchange chromatography on Dowex-50 columns. The protein fractions were dissolved in alkali, precipitated, washed with cold 5% TCA and redissolved with 10% NH_4OH. In all cases radioautograms were used to identify the labeled components of the cold TCA extracts, the hydrolyzates of NA and the hydrolyzates of protein. Aliquots were evaporated to dryness on stainless steel planchets, counted in a Geiger-Muller end-window counter and their radioactivities corrected to infinite thinness.

Materials

Adenine was obtained from Nutritional Biochemicals corporation and used without further purification. 2-^{14}C-glycine (0.43 mc/mmole) was obtained from the Commissariat à l'Energie Atomique France. Uniformly ^{14}C-labeled adenine, guanine and proline were isolated from *E. coli* grown on uniformly labeled glucose (14 mc/mmole). When chromatographed, the purines and amino acids were found to be radiochemically pure.

EXPERIMENTAL RESULTS

The extent of intracellular degradation of NA and protein in growing yeast was determined by studying the kinetics of utilization of metabolic pools. The extent of protein turnover was checked independently by an internal trapping procedure.

a. Kinetics of the utilization of metabolic pools

Previous studies have indicated the presence in yeast of precursor pools for both nucleic acid and protein synthesis[9]. These precursor pools can be preferentially labeled by briefly immersing exponentially growing cells into a medium containing the ^{14}C-labeled precursors. Assuming random mixing of the labeled components with the non-radioactive components of the pool, the kinetics of transfer of the carbon from these precursors to final end products can be predicted in exponentially growing cells.

If a quantity of cells, Q, is growing exponentially, then

$$Q = Q_0 \epsilon^{\alpha t}$$

where Q_0 is the original quantity of cells, Q the number at time t, and α the growth constant. Consider the following biosynthetic sequence in this system:

$$M \rightarrow P \underset{k_2}{\overset{k_1}{\rightleftharpoons}} B \qquad (1)$$

in which M = medium component; P = precursor; B = protein or nucleic acid.

Since the ratios of M, P and B are constant during exponential growth

$$\begin{aligned} P &= P_0 \ \epsilon^{\alpha t} \quad \text{(a)} \\ B &= B_0 \ \epsilon^{\alpha t} \quad \text{(b)} \end{aligned} \qquad (2)$$

When the rates of formation and utilization are proportional to Q, then

$$\frac{dB}{dt} = k_1 P - k_2 B \qquad (3)$$

Differentiating (2) and substituting in (3) gives the relationship between the growth constant, α, and the transfer coefficients k_1 and k_2

$$\alpha = \frac{P_0}{B_0} k_1 - k_2 \qquad (4)$$

The kinetics of utilization of P can be examined by briefly growing the culture in

medium containing radioactive P^* and then transferring the culture to non-radioactive medium at $t = 0$. Let us define P^* and B^* as the amount of radioactivity per bacterium. During steady-state growth, the total radioactivity remains constant and

$$P^* + B^* = P_0^* \, e^{-\alpha t} \tag{5}$$

The dilution of P^* during exponential growth will be

$$\frac{dP^*}{dt} = -(\alpha + k_1) P^* + k_2 B^* \tag{6}$$

providing that there is random mixing between radioactive and non-radioactive components and that $P \gg P_0^*$.

Substituting (5) in (6) and integrating between the limits 0, P_0^* and t, P^* it can be shown that

$$P^* = \frac{k_2}{k_1 + k_2} P_0^* e^{-\alpha t} + \frac{k_1}{k_1 + k_2} P_0^* \, e^{-(k_1 + k_2 + \alpha)t} \tag{7}$$

Multiplying equation (7) by $e^{\alpha t}$, the total pool radioactivity in the culture ($P^* e^{\alpha t}$) will be

$$P^* \, e^{\alpha t} = \frac{k_2}{k_1 + k_2} P_0^* + \frac{k_1}{k_1 + k_2} P_0^* \, e^{-(k_1 + k_2)t} \tag{8}$$

When $k_2 = 0$, then (8) reduces to the form similar to that described by COWIE AND WALTON[10].

$$P^* \, e^{\alpha t} = P_0^* \, e^{-k_1 t} \tag{9}$$

The above analysis requires that the precursor candidate be an end family member of the biosynthetic sequence, as well as a specific precursor of either protein or nucleic acid synthesis. In yeast, arginine fulfills these requirements among amino acids[11], and on the basis of the following observations, guanine was chosen as a precursor for nucleic acids. Radioautographic analysis of cells grown on ^{14}C-adenine reveals that 40% of the adenine is converted to guanine; when the cells are grown on ^{14}C-guanine, no labeling is found in the other bases. Since the ratio of ribosenucleic acid (RNA) to desoxyribosenucleic acid (DNA) is approximately fifteen in this strain (LK2G12), experiments with guanine are largely indicative of RNA behavior.

Exponentially growing cells were exposed to ^{14}C-labeled arginine or guanine for 10 minutes, washed, and resuspended in fresh growth medium. In Fig. 1 and 2, the flow of the labeled cold-TCA-soluble carbon to the protein fractions and to the nucleic acid fractions is compared with predicted plots for various values of k_2 obtained from equation (8). Little of the incorporated carbon is lost to the medium in such experiments. The coincidence of the experimental plot with the predicted plot for $k_2 = 0$ demonstrates that, in agreement with studies in *T. utilis*[10], the formation of protein and NA is essentially irreversible in exponentially growing *S. cerevisiae* LK2G12. Assuming a maximal error of $2\sigma_m$ in the determination of the final $P^* e^{\alpha t}$ in Figs. 1 and 2, k_2 has a maximal limit of 0.01. Therefore k_2 is less than 0.025 and 0.037% of the synthetic rates of protein and nucleic acid respectively.

b. Internal trapping method for measuring protein turnover

This method, recently used by KOCH AND LEVY[12] with *E. coli*, is based on the fact that glycine is a precursor for both nucleic acid purines and for glycine and serine in the

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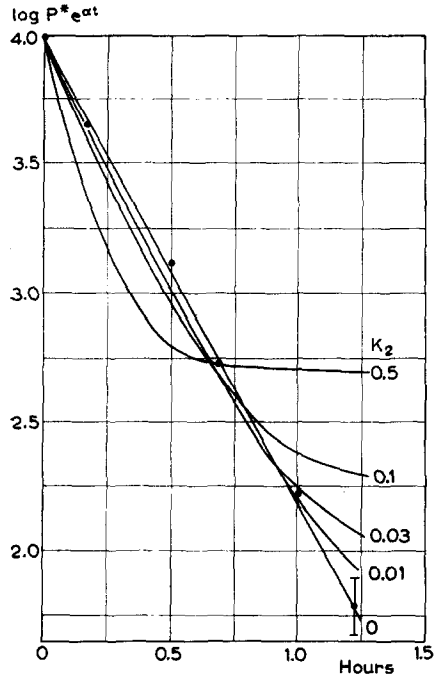

Fig. 1. Kinetics of utilization of ^{14}C-labeled arginine pool. An Erlenmeyer flask containing 100 ml synthetic medium exponentially growing cells (O.D. of 0.500) was inoculated with 3.4 mμmoles totally labeled ^{14}C-arginine. After incubation for 10 minutes at 30° C the suspension was washed, and the cells resuspended in 500 ml of warm synthetic medium. At intervals, 75 ml samples were removed, washed and cold TCA fractions prepared from each. The total pool radioactivity ($P^* e^{\alpha t}$) is plotted against time in hours. The growth rate during this period was 0.453 h^{-1}. The ratio of P_0/B_0 was determined as 0.111 by decarboxylase analysis[9]. From equations (4) and (8), the theoretical curves for various values of k_2 were drawn.

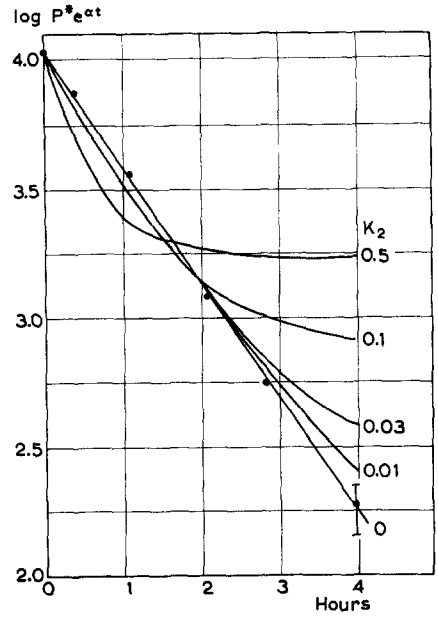

Fig. 2. Kinetics of utilization of ^{14}C-labeled guanine pool. Cells were prepared and treated as described in Fig. 1 employing 10 minute incubation with 1.5 mμmoles totally labelled guanine in 100 ml. The growth constant was 0.340 h^{-1}. B_0/P_0 was calculated from the data (equation 4) assuming $k_2 = 0$. Theoretical curves (equation 8) are drawn for various k_2 values.

proteins of yeast[13]. Under suitable conditions, nucleic acids may be used as an intracellular trapping device for glycine and serine generated by the breakdown of protein. The fact that exogenous purines can suppress *de novo* purine synthesis by yeast[14] and the fact that the latter is restored upon removal of the exogenous purines without affecting the growth rate, make this method experimentally feasible.

The cells were grown in the following stages:

I. In the presence of exogenous purines and 2-^{14}C-glycine. Under these conditions *de novo* synthesis of purines is suppressed.

II. After being washed, the cells were grown in the presence of exogenous purines. This step is required to increase the sensitivity by depressing the labeling of the large free amino acid pool of yeast[9].

III. After again being washed, the cells were grown in a medium devoid of both glycine and purines. At intervals the yeast cells were fractionated and the total isotope content of the pool, the protein, and the nucleic acid purines determined. Any increase

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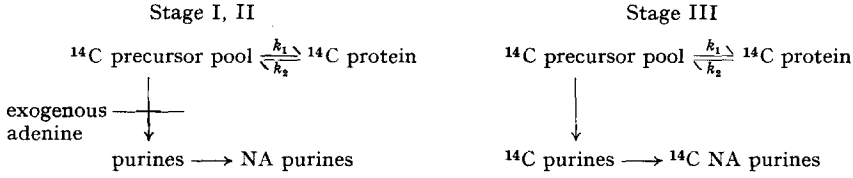
in the nucleic acid purines was attributable to a reutilization of purine intermediates derived from protein breakdown, as indicated in the following scheme:



In accordance with this scheme, the rate of ^{14}C protein (B) breakdown in an exponentially growing culture (equation 1) is

$$k_2 = \frac{q}{B}\frac{dN}{dt} \qquad (10)$$

where dN/dt is the rate of increase in the activity of nucleic acid purines (N) and q is the probability that the fragments derived from the protein are utilized for purine synthesis.

The value of q can be measured from yeast growing in the presence of labeled glycine and in the absence of exogenous purines as the ratio $N/(N+B)$ assuming that under these conditions nucleic acid synthesis is essentially irreversible and that the labeled products of protein degradation are similar to those derived from exogenous 2-^{14}C-glycine. The validity of these assumptions is considered at the end of this section. If t is expressed in hours, the corresponding half-life of proteins (τ 1/2) will be (in days)

$$\tau\ 1/2 = \frac{0.693}{24}(q)(B)\frac{dt}{dN} \qquad (11)$$

The foregoing method is based on changes in the value of q. Before the three-stage experiment can be used to measure turnover, it is necessary first to determine the value of q, the extent to which it is suppressed by exogenous purines, and the rapidity with which q returns to its steady-state value during stage III in the absence of exogenous purines. The following experiments deal with these points in this strain.

The steady-state value of q was determined by growing cells in the presence of 2-^{14}C-glycine and in the absence of exogenous purines. The cells, harvested during

TABLE I

INHIBITION OF PURINE SYNTHESIS FROM GLYCINE BY EXOGENOUS ADENINE

Adenine μmoles/10 ml	*Protein* c.b.m.	*TCA-soluble* c.p.m.	*NA purines* c.p.m.	*Total* c.p.m.	*NA purines* %
0	8,250	1,800	6,020	16,070	38.1
1.45	13,200	2,120	975	16,295	6.0
7.25	13,530	2,200	707	16,437	4.3
29.0	13,800	2,410	774	16,984	4.7

The Erlenmeyer flasks contained 10 ml of synthetic medium containing 14.5 μmoles of 2-^{14}C-glycine, various concentrations of adenine and yeast to an optical density of 0.002. The suspensions were aerated at 30°C until they reached an O.D. of 0.410, centrifuged and washed. The total isotope contents of the cold TCA fraction, protein and isolated nucleic acid purines (see methods for separation) were determined. There was no appreciable labeling of pyrimidines or other cell fractions.

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exponential growth, contain the following isotope distribution (Table I): protein (51.3 %), nucleic acid purines (38.1%) and precursor pool (10.6%). Therefore the normal probability that 2-^{14}C-glycine, or its degradation products, can be used for purine synthesis is 0.43.

The influence of exogenous adenine on q is shown in Table I. Exogenous adenine reduces the incorporation of radiocarbon into nucleic acid purines. Increasing the concentration of exogenous adenine beyond 29 μmoles/10 ml did not further reduce the isotope incorporation into the nucleic acid purines. At the higher concentrations of adenine, the internal pool of free adenine was proportional to the exogenous adenine concentration within the limits examined, whereas the adenine nucleotide pool was relatively unchanged. In experiments in which growing cells were exposed for 10 min to low concentrations of ^{14}C-adenine (2 mμ moles/10 ml), the radioactivity of the cold TCA fraction was found only in the adenine nucleotides. A similar expandable free adenine pool has been observed[14] in *T. utilis*.

The adenine pool is not lost by washing with water or medium. When cells with high adenine pools are placed in fresh growth medium, the adenine pool, as seen by examination of radioautographs, is gradually incorporated into the adenine nucleotide pool and into nucleic acid adenine. BOLTON and coworkers[14] have established by kinetic and isotopic competition experiments with *T. utilis* that nucleic acid adenine is directly incorporated from the phosphorylated adenine pool, which in turn can derive its adenine from *de novo* synthesis or from the expanded adenine pool. It is therefore not surprising that there may be maximal limits for the effective inhibition of adenine synthesis by the expanded adenine pool. Thus in the present experiments, using 2-^{14}C-glycine (Table I), although the adenine pool is not saturated in cells grown in the presence of 29 μmoles adenine/10 ml, near maximal inhibition of *de novo* purine synthesis is observed at 1.45 μmoles exogenous adenine/10 ml. A separation of the nucleic acid purines from these experiments on Dowex-50 columns revealed that the majority of the isotope resided in guanine, indicating that a pathway of guanine synthesis other than via adenine may exist in yeast as well as in *E. coli*[12].

The presence of an expanded adenine pool reduces the probability, q, that isotopically labeled serine and glycine released by protein breakdown will be used for purine synthesis. When cells are grown in the absence of exogenous adenine, the expanded adenine pools are depleted via their phosphorylation and incorporation into nucleic acids. In order to determine the dilution of the expanded adenine pool required to increase q to its original value, the following experiment was conducted. Cells were grown in the presence of 4 μmoles adenine/10 ml, washed and transferred to medium lacking adenine. At intervals aliquots were removed, grown for 10 minutes in the presence of 5 μmoles 2-^{14}C-glycine/10 ml and fractionated for protein and nucleic acid purines. The results, Fig. 3, show that a maximum probability of 0.43 is reached within 40 min of exponential growth. Since stage III was extended for periods up to 12 hours, a short delay in reestablishing a q of 0.43 does not seriously affect the calculation of k_2.

The above experiments provide and justify the use of a q value of 0.43 in equation 10. In order to measure k_2 from this equation, three-stage experiments were carried out as shown (experiment A) in Fig. 4. In all cases, stages I and II were analogous to those described above. Where necessary the cultures were diluted sufficiently to maintain continuous exponential growth. The isotope distributions at intervals during

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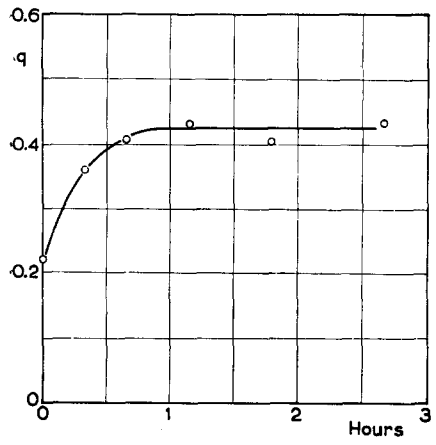

Fig. 3. Dilution of the expanded adenine pool during exponential growth. See text for details. The effect of the adenine pool in suppressing purine synthesis from 2-^{14}C-glycine is measured by the probability (q) that radioactive components from glycine are incorporated into nucleic acids c.p.m. nucleic acid purines/(c.p.m. nucleic acids + c.p.m. protein).

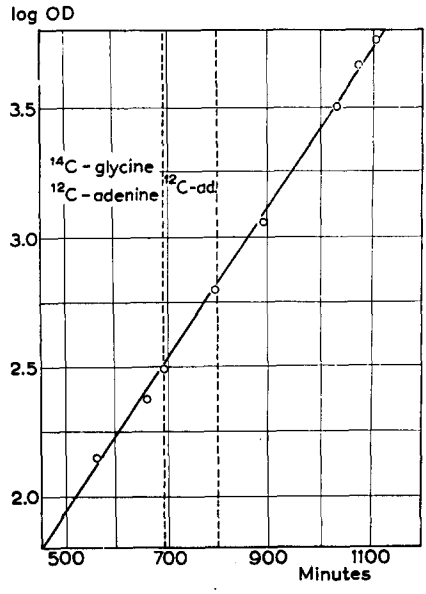

Fig. 4. Growth in the presence and absence of exogenous adenine. An Erlenmeyer flask containing 100 ml synthetic medium with 14.5 μmoles 2-^{14}C-glycine and 72.5 μmoles of adenine was inoculated with yeast to a density of 0.0014 and aerated at 30°C. At the end of Stage I, the culture was centrifuged, washed, resuspended in 100 ml medium with 72.5 μmoles of adenine, and incubated as previously. At the end of Stage II, the suspension was again harvested, washed and resuspended to original volume. 20 ml was removed for analysis (A), and 40 ml diluted to 200 ml with synthetic medium and aerated at 30°C during Stage III. After 87 minutes 100 ml sample was removed (B), 100 ml fresh medium added, and the suspension incubated as previously (C).

stage III are shown in Table II. The average rate constant for protein degradation (k_2) during exponential growth (Table II) was $2.87 \cdot 10^{-4}$. From equation (11), this corresponds to a half-life of yeast proteins of 18 days.

The internal trapping procedure is based on the following assumptions from which a number of possible errors, in addition to those described elsewhere[12], may arise:

1. A closed system.

2. Irreversible nucleic acid synthesis.

3. Similarity of labeled products derived from exogenous 2-^{14}C-glycine and from protein breakdown.

4. Homogeneity of protein turnover.

The validity of these assumptions is supported by the following considerations:

1. Within the limits of detection, the total radioactivity at the end of stage I is recovered in the various fractions at the termination of the experiment. During stages II and III, there is no measurable loss of radioactivity to the medium or to the gas phase.

2. If nucleic acid breakdown occurred during exponential growth, a portion of the radioactivity originally trapped in nucleic acid purines from protein would be released to the cold TCA soluble fraction, thereby giving too low a measure of k_2. However, from the data of Fig. 1, the rate of breakdown of nucleic acids must be less than

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TABLE II

PROTEIN TURNOVER IN GROWING YEAST

Experiment	*Stage II hours*	*Stage III hours*	*B c.p.m.*	*N c.p.m.*	*dN c.p.m.*	$k_2 \cdot 10^4$
A	2	0	$2.66 \cdot 10^5$	$1.47 \cdot 10^4$		
		1.45		$1.49 \cdot 10^4$	200	2.2
		5.17		$1.52 \cdot 10^4$	500	1.6
B	2	0	$1.20 \cdot 10^6$	$6.50 \cdot 10^4$		
		5.75		$7.10 \cdot 10^4$	6,000	3.8
C	3	0	$3.06 \cdot 10^5$	$1.62 \cdot 10^4$		
		4.1		$1.69 \cdot 10^4$	700	2.4
D	5	0	$1.11 \cdot 10^6$	$6.06 \cdot 10^4$		
		10		$5.95 \cdot 10^4$	8,900	3.4
		12		$6.25 \cdot 10^4$	11,900	3.8
					average	2.87

At intervals during stage III, cells were fractionated for nucleic acid purines (N) and protein (B) as described in text. The apparent rate constant for protein degradation k_2, was calculated from equation (10): $q/B \cdot dN/dt$.

0.01—a rate that could not significantly alter the observed k_2 for protein breakdown.

3. When cells of *S. cerevisiae* LK2G12 are grown in 2-^{14}C-glycine in the absence of exogenous purines, the isotope is found predominantly in the protein, nucleic acid, and cold TCA fractions. Chromatographic analysis of the protein fraction from these cells shows that glycine and, to a lesser extent, serine contain most of the radioactivity. In similar radioautographs of chromatograms of nucleic acid hydrolyzates, only adenine and guanine contained radioactivity. A time course of the incorporation of 2-^{14}C-glycine into these components of the cold TCA fraction showed that glycine and adenine nucleotides appeared as the first radioactive components. In later samples, guanine nucleotides and serine became radioactive. Similar results were observed when the time courses of incorporation of these components into the protein and nucleic acid fractions were followed. In no case was there any extensive labeling of other cell fractions. Thus, the isotope distribution in *S. cerevisiae* LK2G12 is analogous to that in other yeast strains where 2-^{14}C-glycine is both a precursor of C-2 and C-5 of adenine and guanine[13] and is convertible to serine[11].

4. The proteins of resting yeast cells are probably heterogenous in their stability[5]. If a similar heterogenity also existed in growing yeast, it would be difficult to detect because an extended stage II of 2–5 hours duration is used to deplete the pool of radioactivity and 40 min are required in stage III to reestablish a q of 0.43. It is difficult to improve the method by using earlier samples since in the experiments of short duration, (A and C, Table II), $\Delta N/N$ ranges within limits (0.02 to 0.04) expected from experimental errors. In experiments of longer duration, (B and D, Table II) however, $\Delta N/N$ values (0.09 to 0.24) are greater than experimental errors, and the overall k_2 can be measured more accurately. Because the percentage of radioactivity in nucleic acid purines from stage I (Table I) is in close agreement with the percentage found at the end of stage III (Table II), any proteins with high turnover rates that may be present must constitute only a small fraction of the total proteins of yeast.

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DISCUSSION

Since SCHOENHEIMER[2] advanced his concept of a "dynamic state", there has been a continual interest in protein and nucleic acid turnover in Metazoa and micro-organisms. Since demonstrable intracellular turnover is found in resting yeast cells[5], it is of particular interest to determine whether such turnover occurs in growing yeast. Both exponentially growing and resting yeasts contain nucleotide and amino acid pools[9]. During exponential growth, precursors are withdrawn in a random manner for protein and nucleic acid synthesis. These findings in yeast are in agreement with those of COWIE AND WALTON[10], and oppose the concept of an assembly-line synthesis of these cellular components. The specific turnover rate of these precursor pools depends upon their respective size and upon the quantity required for macromolecule synthesis. In the growing cell these precursors are incorporated into essentially stable end products, since the k_2 values for breakdown are $2.87 \cdot 10^{-4}$ h^{-1} for protein and less than 10^{-2} h^{-1} for nucleic acids. In resting cells the minimal rates of breakdown of protein and nucleic acid were $6.6 \cdot 10^{-3}$ h^{-1} and $1.5 \cdot 10^{-3}$ h^{-1} respectively[5]. Thus although the inaccuracies of measurement of nucleic acid turnover in growing yeast do not permit a critical comparison with resting yeast, the rate of protein breakdown is at least 23 times higher in resting than in growing cells. Protein and nucleic acid stability in exponentially growing yeast is therefore analogous to that of other organisms[12, 15, 16]. These findings not only reject the unlikely possiblilty that the large pools in yeast result from a balance between precursor synthesis and macromolecule breakdown, but also indicate that the amino acid exchange-incorporation reactions are of minor importance in growing yeast, at least for glycine, serine and arginine.

In yeast, the synthesis of α-glucosidase and probably protein in general is coupled with RNA synthesis[9]. When, by stimulating the synthesis of cell proteins, the source of precursors for RNA synthesis (nucleotide pool) is depleted in partially induced yeast, α-glucosidase synthesis is suppressed. It has been argued that the presence of an enzyme-forming system, presumably containing specific RNA, is not sufficient to maintain enzyme formation, since the RNA involved in the synthesis of these enzymes may be unstable. Thus the RNA formed in the early stages of induction would not be available, and its resynthesis would be coupled to enzyme synthesis. The results on nucleic acid turnover indicate that in growing yeast RNA is a stable end product either under constitutive synthesis (Fig. 1) or under conditions of induction. In the latter case, exponential decrease in ^{14}C guanine from the nucleotide pool was observed when induced cells were transferred to inducer-free growth medium. From these experiments, it would appear that in the growing yeast, no appreciable quantities of nucleic acid are degraded to units (nucleotides) which can be used for resynthesis, because if such were the case, one would expect them to be trapped in the nucleotide pool and to lead to non-exponential kinetics of nucleotide utilization.

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SUMMARY

A study of the utilization of individually ^{14}C-labeled amino acid and nucleotide pools in exponentially growing yeast indicated that precursors are converted essentially irreversibly into proteins and nucleic acids. The maximal breakdown rates for each were 0.01 h^{-1}. Employing a more sensitive method, involving an internal nucleic acid purine trap for protein degradation products, the protein breakdown rate was estimated at $2.8 \cdot 10^{-4}$ h^{-1}. The proteins therefore have an average half-life of 18 days or longer in cells dividing every 90 minutes.

The data indicate that protein breakdown in growing cells is only 4% of that in resting cells. The significance of the difference in protein and possibly nucleic acid stabilities of growing and resting cells is discussed.

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