[12] Bloch, D. P., and H. Y. C. Hew, *J. Biophys. Biochem. Cytol.*, **7**, 515–532 (1960).
[13] *Ibid.*, **8**, 69–81 (1960).
[14] Butler, J. A. V., P. Cohn, and P. Simson, *Biochim. Biophys. Acta*, **38**, 386–388 (1960).
[15] Horn, E. C., *Anat. Rec.*, **137**, 365 (1960).
[16] Supplied by C. H. Mumley, Alberg, Vermont.
[17] Moore, J. A., *J. Exptl. Zool.*, **101**, 173–220 (1946).
[18] Miller, G. L., and R. H. Golder, *Arch. Biochem.*, **29**, 420–423 (1950).
[19] Anderson, N. G., R. E. Canning, M. J. Anderson, and R. H. Shellhamer, *Exptl. Cell Res.*, **16**, 292–308 (1959).
[20] Karasaki, S., *Embryologia*, **4**, 247–272, 273–282 (1959).
[21] Palade, G. E., and P. Siekewitz, *J. Biophys. Biochem. Cytol.*, **2**, 171–198 (1956).
[22] Panijel, J., *Actualities Sci. et Ind.*, No. 1150 (1951).
[23] Rounds, D. E., and R. A. Flickinger, *J. Exptl. Zool.*, **137**, 479–500 (1958).
[24] Gregg, J. R., and R. Ballentine, *J. Exptl. Zool.*, **103**, 143–168 (1946).
[25] Yamada, T., in *Advances in Morphogenesis*, ed. M. Abercrombie and Jean Brachet (N. Y.. Academic Press, 1961), vol. 1, pp. 1–53.
[26] Zeller, C., *Arch. f. Entwmech.*, **148**, 311–335 (1956).
[27] Chen, P. S., *Exptl. Cell Res.*, **21**, 523–534 (1960).
[28] Moore, Betty C., *J. Morphol.*, **101**, 227–274 (1957).
[29] King, T. J., and R. Briggs, in *Genetic Mechanisms: Structure and Functions*, Cold Spring Harbor Symposia on Quantitative Biology, vol. 21 (1956), pp. 271–290.
[30] Hoff-Jorgensen, E., and E. Zeuthen, *Nature*, **169**, 245 (1952).
[31] Sze, L. C., *J. Exptl. Zool.*, **122**, 577–601 (1953).
[32] Finamore, F. J., and G. Volkin, *Exptl. Cell Res.*, **15**, 405–411 (1958).
[33] Brachet, J., *Arch. Biol.*, **51**, 167–202 (1940).
[34] Moore, J. A., *Develop. Biol.*, **2**, 535–550 (1960).
[35] Cruft, H. J., C. M. Mauritzen, and E. Stedman, *Nature*, **174**, 580–585 (1954).
[36] Leslie, I., *Nature*, **189**, 260–268 (1961).
[37] Briggs, R., and T. J. King, these PROCEEDINGS, **38**, 455–463 (1952).
[38] Briggs, R., and T. J. King, *J. Morphol.*, **100**, 269–312 (1957).
[39] Wittek, M., *Arch. Biol.*, **63**, 133–198 (1952).
[40] Yamamoto, K., *J. Fac. Sci., Hokkaido Univ.*, Ser. 6, **12**, 375–390 (1956).

---

*METABOLIC PROPERTIES OF A RIBONUCLEIC ACID FRACTION IN YEAST**

BY YOSHIJI KITAZUME,† MARTYNAS YČ S, AND W. S. VINCENT‡

DEPARTMENTS OF MICROBIOLOGY AND ANATOMY, STATE UNIVERSITY OF NEW YORK, UPSTATE MEDICAL CENTER, SYRACUSE, NEW YORK

Communicated by C. W. Metz, December, 1961

In a previous communication, Yčas and Vincent[1] reported that if exponentially growing yeast is presented with a short pulse of orthophosphate, P^{32}, the ratios of total counts in the 2′ and 3′ ribonucleotides obtained by alkaline hydrolysis of the RNA[2] are similar to the ratios of the corresponding deoxyribonucleotides in yeast DNA (uracil being regarded as corresponding to thymine). In exponentially growing yeast, labeled phosphorus initially enters an unstable RNA fraction having a composition similar to the corresponding DNA. Volkin, Astrachan, and Countryman[3] had demonstrated earlier that such a fraction exists in phage-infected bacteria, where newly formed RNA appears to mimic the composition of phage DNA. Subsequently, other workers have reported the existence of similar RNA fractions

in other forms.[4] We prefer to refer to this RNA fraction as "dRNA," a terminology which relates this RNA only to its apparent resemblance in composition to DNA. Such terminology avoids implications of function for which no adequate experimental demonstration as yet exists.

The determinations which Yčas and Vincent had made on dRNA indicate that it has a short lifetime in growing cells.[1] In order to examine this fact against the background of the literature which relates RNA synthesis and the synthesis of proteins, we have studied the kinetics of synthesis of the dRNA fraction in yeast cultures which had been placed in differing metabolic states. The results of these studies, presented below, indicate that there is no obvious relationship between dRNA synthesis and protein synthesis, that in the absence of growth there is an accumulation of dRNA, and that dRNA is an obligatory precursor of the bulk of cellular RNA.

Materials and Methods.—The strains of yeast (*Saccharomyces cerevisiae*) used were as follows: 59RA, a cytochrome-deficient strain, previously described[1]; A-589A, requiring histidine, methionine, isoleucine, and tryptophan; and A-589B, requiring uracil and adenine, both from Herschel K. Roman; 14964A, requiring lysine, from Carl Lindegren. Media: natural growth medium; as previously described[1]; synthetic growth medium of the following composition: glucose, 30 gm; KH_2PO_4, 100 mg; $MgSO_4 \cdot 7H_2O$, 250 mg; KCl, 100 mg; NH_4Cl, 2 gm; L-asparagine, 2.5 gm; thiamine HCl, 0.5 mg; pyridoxine, 0.25 mg; calcium pantothenate, 0.25 mg; inositol, 20 mg; biotine, 0.15 mg– all per one liter of 0.02 *M* citrate buffer, pH 5.0. Other growth requirements were added as needed. Incubation of cells in buffer was performed in medium of the following composition: glucose, 30 gm; KCl, 0.5 gm; $MgSO_4.7H_2O$, 0.2 gm in one liter of 0.01 *M* tris-(hydroxymethyl)-aminomethane adjusted to pH 6.8 with HCl. All incubations were under aerobic conditions at 30°C. Optical density of cultures was measured at 650 mμ using a Beckman model DU spectrophotometer. Extraction and deproteinization of RNA was performed with hot NaCl solution and octanol-chloroform as previously described.[1] RNA was hydrolyzed with 0.5 *N* KOH for 17 hr at 30°C. Nucleotides were separated on columns of Dowex-1, 8% cross linkage, in a chloride system.[4] The apparent specific activity of the P^{32}-labeled 3′ isomer of adenylic acid was usually higher than that of the 2′ isomer after this separation. Electrophoresis on paper[5] showed that this is due to contamination of the 3′ isomer with highly labeled inorganic phosphate and that the true specific activities of both isomers are equal and correspond to the specific activity of the 2′ isomer separated by ion exchange. We therefore present only the values of the specific activities of the 2′ isomer of adenylic acid. To improve the accuracy of our results, we have generally worked with quantities of RNA sufficient to give 0.5 to 1.0 mg of each nucleotide and optical densities at 260 mμ of upward of 0.5. Spectrophotometry and counting have been previously described.[1] All specific activities are expressed as counts/min/μM. Total uptake of labeled material into cells was determined by filtering a suitable aliquot of cultures through "Millipore" filters,[6] washing the retained cells with cold 0.1 *N* perchloric acid, mounting the filters on planchets, and counting after drying. Total uptake, therefore, refers to uptake by cells in a given volume of culture medium. Other details of methods are described in the legends and the text.

Experimental Results.—We have studied synthesis of RNA in yeast under two

different conditions: (1) in glucose-tris buffer and (2) in exponentially growing cultures.

1. *RNA synthesis in buffer:* (a) *Amount of RNA synthesized:* If exponentially growing yeast is washed and then suspended in glucose-tris buffer, further net growth is of course not possible once endogenous pools are exhausted. However, if C^{14}-labeled uracil is added to the buffer, there is a considerable uptake of label not extractable with cold 0.1 *N* perchloric acid. The uptake is rapid initially and then levels off or continues at a much reduced rate (Fig. 1). Decrease of uptake

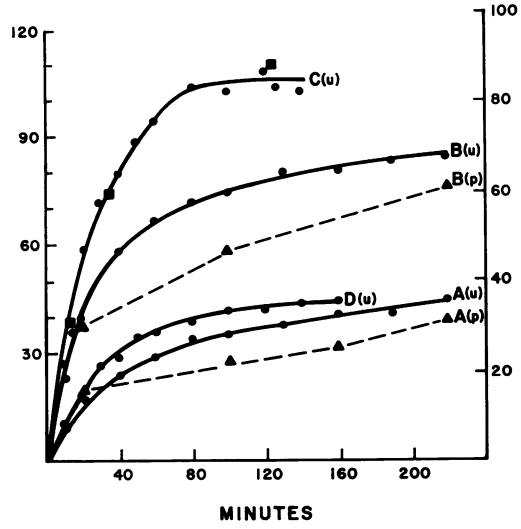


FIG. 1.—Uptake of uracil-C^{14} and orthophosphate-P^{32} by yeast 59RA in buffer. Left ordinate—specific activity $\times 10^3$ of P^{32} in extracted RNA (curves A (p) and B (p), from data of experiments A and B in Table 3). Right ordinate—total uptake of uracil-C^{14}, arbitrary scale. Curves A(u) and B(u) show the total uptake of uracil-C^{14} by aliquots of cultures used to measure uptake of P^{32} in experiments A and B in Table 3. Curve D(u) shows a similar total uptake by an aliquot of culture used in experiment on P^{32} uptake in Table 1. Curve C(u) is total uptake of uracil-C^{14} in experiment on uracil uptake shown in Table 1. Black squares superimposed on curve C(u) are the calculated amount of synthesis of RNA from uptake of uracil-C^{14} into the isolated uridylic and cytidylic acids isolated from extracted RNA (Table 1, arbitrary scale).

with time is not due to exhaustion of uracil-C^{14} from the medium, since only a few per cent of the amount added is taken up by the cells. To demonstrate that this uptake represents incorporation of uracil into RNA, we have simultaneously measured total uptake of uracil into cells, uptake into RNA extracted from such cells, and the specific activity of uracil in the medium. If the specific activity of newly synthesized RNA is the same as that of the precursor in the medium, from the latter two measurements it is possible to calculate the actual synthesis (net or turnover) of RNA in buffer. Such calculations are presented in Table 1 and show that synthesis is about 2 per cent of the amount initially present. The same result is obtained using labeled orthophosphate as a precursor (Table 1). The curve representing RNA synthesis as determined from the radioactivity of uracil-labeled RNA is virtually superimposable on the curve of total uptake of uracil (Fig. 1, curve C(u)). Thus, total uptake of label into cells is proportional to and is a valid measure of RNA synthesis.

TABLE I

UPTAKE OF URACIL-C^{14} AND ORTHOPHOSPHATE-P^{32} INTO NUCLEOTIDES OF EXTRACTED RNA

		UR-C^{14}					P^{32}		
Time		c/min/ μM	X Mole fraction	Total counts per μM nucleotide in RNA	Ratio SA CY/UR	Per cent synthesis	c/min/ μM	Calculated composition	Per cent synthesis
15′	CY	36.7	7.3						
				30.6	0.42	0.80			
	UR	87.5	23.3						
35′	CY	83.5	16.7						
				59.6	0.53	1.47			
	UR	159.0	42.9						
125′	CY	133.0	26.6				577	0.239	
				88.7	0.58	2.15			
	UR	230.0	62.1				497	0.275	
	AD	—					568	0.298	2.0
	GU	—					340	0.189	
Specific activity in medium		10,953					24,947		

Yeast 59 RA, harvested during exponential growth and incubated in tris buffer with glucose. Per cent synthesis calculated on the assumption that newly synthesized material has the same specific activity as precursor in medium. Total uptake of uracil-C^{14} into cells in this experiment shown as curve C(u) in Figure 1. Curve D(u) in the same figure shows total uptake of uracil-C^{14} by an aliquot of the suspension used for the P^{32} uptake experiment presented in this table. Nucleotides obtained by alkaline hydrolysis.

To determine whether uptake of uracil into cells represents a net synthesis or turnover of a small fraction, we have suspended cells in glucose-buffer containing cold uracil in excess. Labeled uracil was then added to aliquots at different times and total uptake into the cells determined. As can be seen from Figure 2, the later

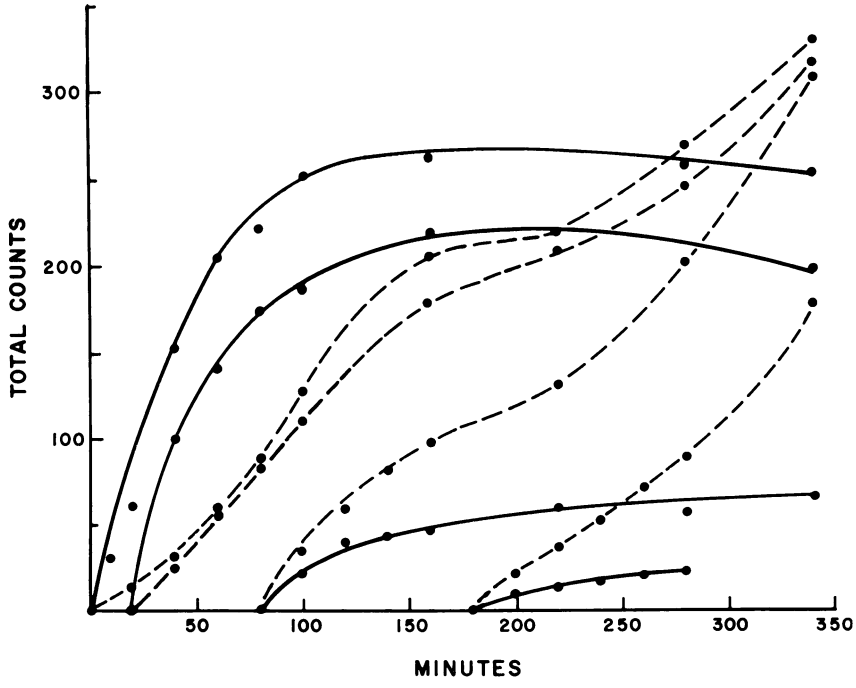


FIG. 2.—Uptake of uracil-C^{14} (solid lines) and proline-C^{14} (dashed lines) into yeast 59 RA, suspended in buffer with 4 μg/cc uracil-C^{12} and 8 μg/cc DL-lysine-C^{12}. Labeled precursors added to aliquots at 0, 20, 80, and 180 min.

the addition of labeled uracil, the less are both the initial rate and the maximum uptake. There is, however, some incorporation of label added later even when the amount of isotope in cells to which isotope was added to zero time remains constant or even falls slightly. We interpret this result to mean that uptake of uracil into RNA in buffer represents primarily net synthesis but that the RNA formed is somewhat unstable and shows some turnover. As we demonstrate below, under certain conditions this RNA fraction can be markedly unstable. Net synthesis, however, seems to be the dominant phenomenon in buffer.

(b) *Relationship of RNA synthesis to amino acid incorporation:* Although there appears to be little turnover of RNA in buffer, an apparent turnover of protein can be readily demonstrated. As shown in Figure 3, labeled lysine-C^{14} is rapidly in-

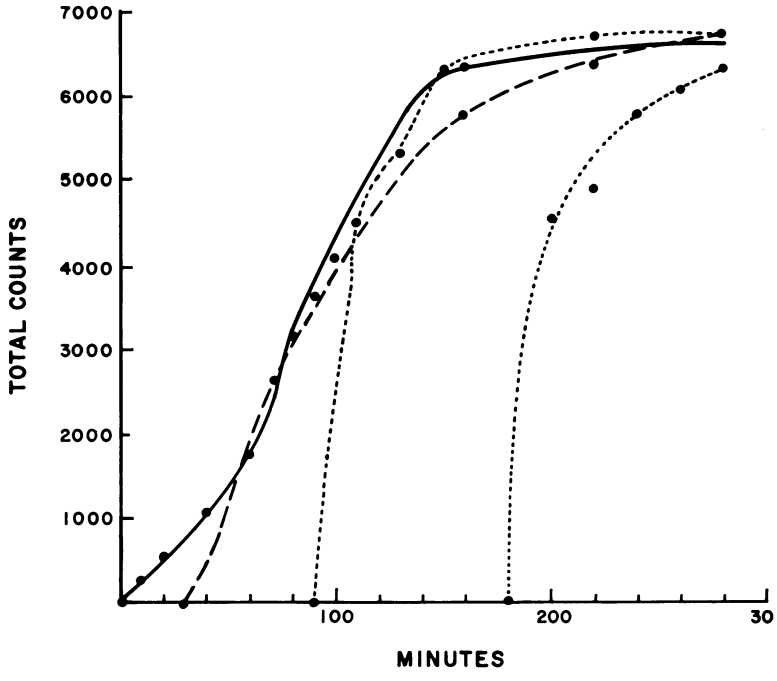


FIG. 3.—Incorporation of DL-lysine C^{14} into yeast 59 RA in buffer with 10 μg/cc of DL-lysine-C^{12}. Isotope added at 0, 30, 90, and 180 min.

corporated. The label is not removed by incubating the cells for 15 min in carbonate buffer at pH 11.5, as would be the case if the amino acid formed an ester linkage with "soluble" RNA.[7] Unlike uracil, neither the rate nor the maximum incorporation attained decreases with time, and the shape of the curve of incorporation suggests that there is a specific protein fraction in yeast which is undergoing turnover. Proline incorporation (Fig. 2) shows somewhat different kinetics, reaching an apparent plateau and then increasing again. This presumably reflects the fact that proline, unlike lysine, can serve as a general source of nitrogen for the cell and thus permit some growth. It will be noted that in buffer there is no obvious correlation between the synthesis of RNA and incorporation of amino acids.

The size of the RNA fraction formed in buffer depends to some degree on the

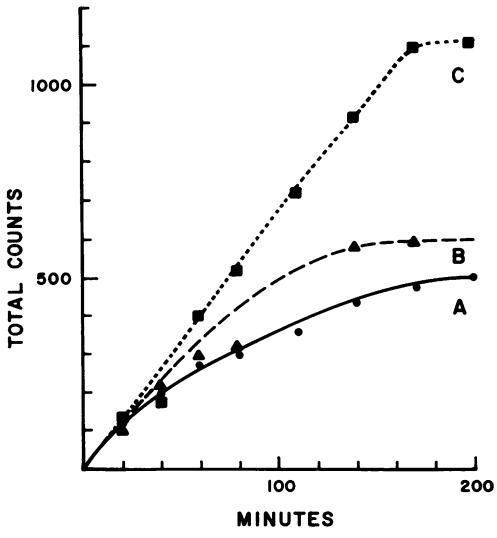


FIG. 4.—Uptake in buffer of uracil-C^{14} by yeast A-589A, requiring histidine, isoleucine, tryptophan, and methionine for growth. A—NO amino acids; B—100 μg/cc isoleucine; C—100 μg/cc each of isoleucine, histidine, tryptophan, and methionine.

availability of amino acids. Using a mutant strain of yeast (A-589 A) requiring isoleucine, histidine, methionine, and tryptophan for growth, we find that uptake of uracil takes place in buffer even in the absence of these amino acids. However, there is a marked stimulation by added isoleucine and even more by all four of these amino acids added together (Fig. 4). Since it has been shown that synthesis of RNA by yeast requires the presence of amino acids,[8] the formation of RNA by this mutant strain is presumably possible because of the existence of a pool of free amino acids, a pool that it is difficult to deplete completely in yeast.[9]

(c) *Stability of RNA to external media:* In distilled water, the fraction of RNA formed in buffer shows a marked instability. Cells briefly labeled with uracil-C^{14} in buffer and transferred to distilled water lose a large part of the incorporated radioactivity, and this loss is even greater if ribonuclease is present in the medium (Fig. 5). We have no information, however, whether this is due to the

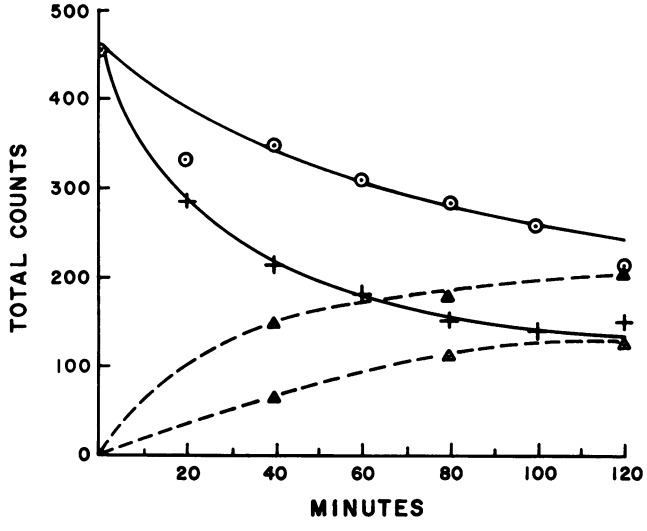


FIG. 5. Loss of incorporated uracil-C^{14} in distilled water. Yeast 59 RA preincubated in buffer with uracil-C^{14} for 30 min., washed in cold and suspended in distilled water with and without ribonuclease. Total counts per cc of medium. Time in min from suspension of cells in water.
⊙—cells in H_2O; +—cells in H_2O with ribonuclease (10 μg/cc); △—supnatanter H_2O; ▲—supernatant with ribonuclease.

enzymatic activity of ribonuclease. The point of interest is that the breakdown is one preferentially involving the fraction of RNA that is formed in buffer. This is demonstrated by the data in Table 2, which show that the specific activity of the RNA remaining in the cells after suspension in distilled water decreases. This could not occur if all fractions of RNA were being destroyed at the same rate. Clearly, so far as susceptibility to destruction in distilled water is concerned, the fraction of RNA formed in buffer is in a different state from the bulk of cellular RNA.

TABLE 2

REMOVAL OF P^{32}-LABELED RNA FROM CELLS WITH DISTILLED WATER AND RIBONUCLEASE

	Counts/min/μM				
	Experiment 1			Experiment 2-*	
	Initial	In H_2O	In H_2O with ribonuclease	Initial	In H_2O with ribonuclease
CY	24120	15770	13445	15215	9083
AD	26464	15668	13485	18111	10466
UR	24936	13531	11361	16584	9634
GU	18080	10759	10874	11927	8275
			Calculated composition*		
CY	0.21	0.23	0.22	0.20	0.20
AD	0.29	0.29	0.28	0.30	0.29
UR	0.29	0.27	0.25	0.29	0.28
GU	0.21	0.21	0.24	0.21	0.24

Yeast 59 RA washed, suspended in buffer with glucose and P^{32} for 30 min, then washed and resuspended in distilled water with and without ribonuclease. After 60 min incubation at 30°C, yeast killed with 0.1 *N* PCA, RNA extracted, hydrolyzed with alkali and specific activity of nucleotides determined. Ribonuclease concentration 10 mg./l.

* See text.

Concomitantly with the breakdown of this fraction in distilled water, there is an appearance in the medium of material absorbing light in the ultraviolet (compare reference 10). Breakdown is presumably an enzymatic process, since we have found that it does not occur if cells are suspended in distilled water at 0°C.

(d) *Apparent composition of RNA synthesized in buffer:* We have determined the apparent composition of this fraction by labeling it in buffer with P^{32}, extracting the RNA, and determining the distribution of label in the nucleotides obtained by alkaline hydrolysis. Multiplying the specific activity of each nucleotide by its mole fraction in total cell RNA, we obtain the total counts in each nucleotide and thus the apparent composition of the RNA fraction which is formed in the presence of label. These results are presented in Tables 1, 2, and 3. For at least up to 120 min of incubation in buffer with P^{32}, the calculated composition of this fraction is similar to yeast DNA (Table 3) and quite different from the bulk of cellular RNA (Table 4). We conclude that in buffer the RNA formed does not consist of all the RNA species that may be present in the cell but is predominantly, perhaps exclusively, dRNA.

It should be noted, however, that the calculated composition of RNA formed in buffer deviates slightly from the reported composition of yeast DNA. The mean values of twelve determinations (Table 4) show that the quantities of Ad and Ur are lower, and those of Gu and Cy are higher, than would be expected if dRNA were a faithful copy of DNA. Since the quantities of the complementary nucleotides Ad, Ur and Cu, Cy are virtually equal, it may be that the deviation of dRNA from the reported DNA composition is real.

(e) *Fate of RNA previously synthesized in buffer when transferred to growth media:*

TABLE 3

UPTAKE OF P^{32} BY YEAST 59 RA UNDER VARIOUS CONDITIONS

Experiment / Nucleotide	A			B			C			D		
	t	a	b	t	a	b	t	a	b	t	a	b
2′ + 3′ CY	20′	61384	0.22	20′	41110	0.22	10′	10246	0.19	10′	9912	0.21
2′ AD		67536	0.29		41230	0.28		12542	0.30		11105	0.29
2′ + 3′ UR		64336	0.29		41537	0.30		11707	0.30		11032	0.31
2′ + 3′ GU		44780	0.20		28504	0.20		8326†	0.21		7211†	0.20
RNA*		59792			37797			10698			9778	
2′ + 3′ CY	100′	93140	0.23	100′	61812	0.21	100′	20579	0.22			
2′ AD		85306	0.27		63165	0.28		20897	0.28			
2′ + 3′ UR		85178	0.28		61269	0.28		17198	0.25			
2′ + 3′ GU		63235	0.21		48362	0.23		16761†	0.25			
RNA*		80785			58297			18869				
2′ + 3′ CY	160′	106047	0.23				160′	16387	0.23	160′	23573	0.22
2′ AD		103075	0.28					14040	0.25		23053	0.25
2′ + 3′ UR		98389	0.28					14528	0.27		22774	0.26
2′ + 3′ GU		75360	0.22					13702†	0.26		22749†	0.26
RNA*		94795						14540			23420	
2′ + 3′ CY	220′	131369	0.23	220′	78096	0.21	220′	16319	0.22			
2′ AD		121994	0.27		83171	0.28		15351	0.26			
2′ + 3′ UR		113897	0.27		79423	0.28		15015	0.27			
2′ + 3′ GU		96505	0.23		65819	0.23		14577†	0.26			
RNA*		117688			76349			15231				

t—time after addition of carrier free $H_3P^{32}O_4$. *a*—counts/min/μM in nucleotide of extracted RNA. *b*—calculated composition of newly formed RNA from total counts (1). Experiments A and B: exponentially growing yeast washed and suspended in tris buffer with glucose. C and D: as A and B, at t = 10 min, a 100 cc solution of 14 gm Bactopeptone, 1.4 gm yeast extract and 1 gm KH_2PO_4 added to yeast suspension (1,400c.c).

* Weighted mean value of specific activity of average nucleotide in RNA.

† 2′ isomer only.

TABLE 4

COMPOSITION OF DRNA

	RNA	dRNA	DNA
AD	0.256	0.286	0.315
UR	0.270	0.287	0.328*
GU	0.271	0.212	0.185
CY	0.202	0.217	0.173

Calculated composition of dRNA formed in buffer (mean value of 12 determinations from Tables 1, 2, and 3) and analytically determined composition of extracted yeast RNA[1] and yeast DNA.[15]

* Thymine.

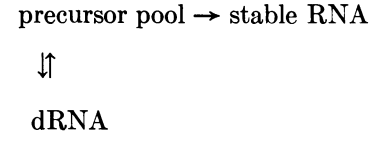
dRNA is relatively stable, but if subsequent growth occurs this fraction of RNA appears to be converted to an RNA with a composition resembling the composition of total cell RNA. We have incubated cells in buffer with carrier-free orthophosphate-P^{32} for 10 min and then added components of a growth medium containing a large excess of unlabeled phosphate. As can be seen from columns C and D of Table 3, after 10 min in buffer the apparent composition of newly synthesized RNA is typical of dRNA. After growth has occurred, however, the distribution of counts in the individual nucleotides indicates that the phosphorus label is now in an RNA fraction which has a composition similar to total cell RNA. However, this demonstration of conversion to another RNA of different composition is not quite unambiguous, since the specific activity of RNA in growth medium continues to increase to almost twice the value at the time the growth medium was added, in spite of the large dilution of P^{32}. It is apparent that the phosphate already present in the cell is used in preference to that available in the medium. Since, however, it has been shown that initial incorporation of phosphate by cells growing in this medium is predominantly into dRNA,[1] it may be presumed that the incorporation

of label after addition of growth medium to buffer is also into dRNA and thus that the change in the distribution of P^{32} among individual nucleotides on resumption of growth indicates a real transfer of material from one RNA fraction to another.

From these experiments, we conclude that in buffer, where growth and especially net protein synthesis is not possible, there is an accumulation of an RNA fraction which has an apparent composition similar to yeast DNA. If growth is resumed, the label incorporated into this fraction appears to be transferred to an RNA with a composition similar to the bulk RNA of the cell.

2. *Kinetics of RNA formation in exponentially growing cultures:* (a) *Kinetic models of RNA synthesis:* From previous results,[1] it is known that in exponentially growing cultures labeled phosphorus initially enters an RNA fraction (dRNA) which is different in composition from the bulk of cellular RNA. Since at least the bulk of the RNA is metabolically stable in growing yeast,[11] one of two kinetic schemes can be invoked to explain this result. dRNA could be a small fraction with a rapid rate of turnover, the products of its breakdown returning to the acid-soluble precursor pool. This can be symbolized as follows:

Scheme 1.



Assuming this scheme to be correct, it is possible to get an approximate estimate of the order of size of the dRNA fraction from composition data. As can be seen in Table 5, it is possible to detect dRNA in exponentially growing cultures by the distribution of label in 3′ nucleotides at least 15 min after addition of P32, when the generation time is 130 min (Table 5, experiment A). With this generation time and assuming a precursor pool of constant specific activity, the amount of stable RNA formed in 15 min must be about 8 per cent of the amount initially present at zero time, so that for dRNA to be detectable by the method used there must be several times (say twice) as much dRNA formed; otherwise, isotope in stable RNA would make the calculated composition of newly formed RNA very different from yeast DNA. One would expect, therefore, that at least 16 per cent and probably considerably more of the total RNA of the yeast cell is dRNA if scheme 1 is correct. This is a very appreciable fraction of the total RNA of the cell, which should be easily detectable by the kinetic experiment we describe below.

Alternatively, dRNA could be a precursor of stable RNA, the conversion not involving the passage of material through the acid-soluble pool. This can be symbolized as:

Scheme 2.

Under scheme 2, the detection of dRNA in exponentially growing cultures would be possible because of a temporal lag between the appearance of isotope in precursor (dRNA) and end product (stable RNA).

Providing certain conditions are met, these two cases can be distinguished by a

TABLE 5

INCORPORATION OF P^{32} INTO THE RNA OF YEAST GROWING EXPONENTIALLY IN SYNTHETIC MEDIUM

Experiment	A		B		C		D	
Yeast	14964A		59RA		69RA		59RA	
OD	0.3		0.5		0.3		0.5	
GT	130		240		312		258	
KH_2PO_4	50		50		1000		1000	
P	2183018		1083000		64780		10490	
	5′	C	5′	C	60′	C	15′	C
CY	20520	0.220	360	0.254	956	0.181	—	—
AD	16470	0.223	337	0.300	1279	0.307	99	—
UR	20848	0.298	277	0.261	1025	0.260	67	—
GU	18026	0.259	197	0.185	985	0.251	54	—
$\bar{M}$	18875		287		1064		73	
	10′		10′		120′		30′	
CY	53904	0.244	1318	0.226	3043	0.196	214	0.219
AD	45914	0.263	1364	0.299	3434	0.280	189	0.245
UR	46862	0.284	1129	0.261	3280	0.280	191	0.260
GU	34377	0.209	919	0.213	2817	0.243	200	0.270
$\bar{M}$	44612		1169		3143		197	
	15′		15′				60′	
CY	75239	0.238	3501	0.228			537	—
AD	70636	0.283	3227	0.267			550	—
UR	66316	0.280	3167	0.275			487	—
GU	46682	0.198	2633	0.230			—	—
$\bar{M}$	63836		3099				523	
							120′	
CY							1336	0.217
AD							1261	0.260
UR							1246	0.270
GU							1159	0.253
$\bar{M}$							1243	

OD—optical density of culture at time of addition of label. $\bar{M}$—weighted mean specific activity of nucleotides in RNA. C—calculated composition. GT—generation time in min. KH_2PO_4—amount in mg/l of incubation medium. P—specific activity of phosphorus in medium. All values are specific activities at times, as indicated after addition of P^{32}.

study of the kinetics of uptake of isotope into the RNA of exponentially growing cultures.

If labeled precursor is added to an exponentially growing culture, and the internal precursor pool of the cells immediately reaches and maintains the same specific activity as the precursor in the medium, the total amount of labeled material incorporated (S) into the stable RNA fraction will increase as $S = S_0\ (e^{kt} - 1)$, where S_0 is the amount of RNA present at time $t = 0$. If there is an unstable fraction as under Scheme 1, there will be an additional initial uptake superimposed on the uptake into the stable fraction. Once the unstable fraction reaches the same specific activity as the precursor pool, the rate of total uptake will be exponential. Experimentally, this will be detected as an initially greater rate of uptake, followed by uptake at the same rate as the rate of growth.

(b) *Pool size and equilibration:* It is first necessary to determine whether the assumptions made in setting up the test are actually met. Figure 6 shows that the response to added cold uracil by RNA synthesis is virtually instantaneous, indicating that the precursor pool is in rapid equilibrium with the uracil in the medium. There remains the question of whether the internal pool of precursor in the cell is of constant specific activity, or whether its specific activity is influenced by a rapid exchange of material with dRNA as in Scheme 1. If such were the case,

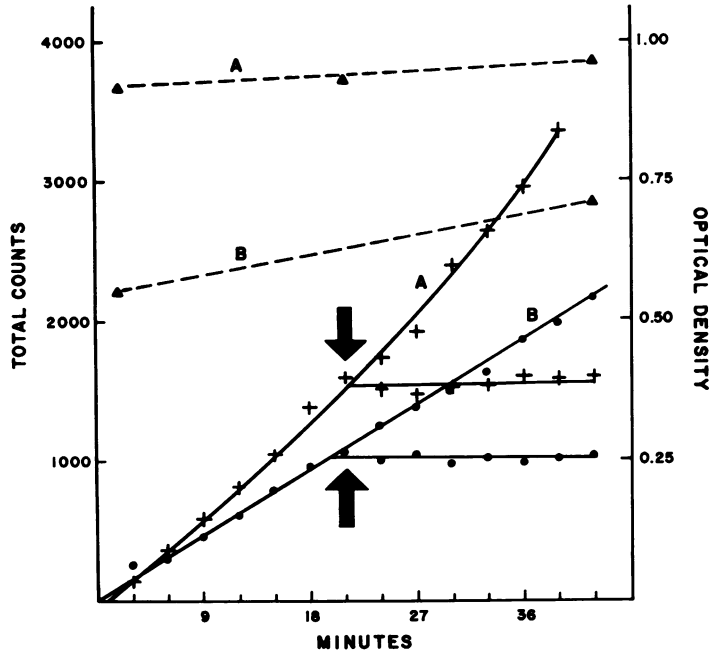


FIG. 6.—Yeast 14964A growing in synthetic medium. Uracil-C^{14} added at zero time. At 21 min, 50-fold excess of uracil-C^{12} added to aliquots. Solid lines—total counts, experiments A and B. Dashed lines—optical density of cultures, experiments A and B.

the assumption of a constant specific activity of the precursor pool would not hold and the kinetic experiment would not yield the information desired.

To elucidate this point, we have determined the specific activity of the precursor pool at short intervals after addition of isotope. To yeast growing in natural medium we added P^{32}. Then, at intervals of 5, 10, and 15 min, samples were chilled and the cells rapidly washed in the cold and hydrolyzed with alkali without preliminary extraction with acid in order to retain the acid-soluble pool in the cells. The hydrolyzate was first fractionated by paper electrophoresis (formate buffer at pH 3.5, 0.1 *M* (see ref. 5) to separate the adenylic acids. The 2′, 3′, and 5′ adenosine phosphates were then separated on a column of Dowex-1 (formate form) by elution with 0.08 *M* formic acid and their specific activities determined. The 2′ and 3′ nucleotides here are derived from RNA and the 5′ are representative of the acid-soluble pool. The results are presented in Figure 7. It will be seen that the precursor 5′ adenosine phosphate reaches a maximum specific activity in less than 5 min and stays constant while the specific activity of the adenylic acid in RNA rises in linear fashion. From this we conclude that the adenylic acid in dRNA is not in rapid equilibrium with the precursor in the acid-soluble pool and comes from a pool with essentially constant specific activity. Since previous results[1] have shown that in this interval of time the ratios of specific activities of all 5′ and 3′ nucleotides derived from RNA by hydrolysis with diesterase and alkali respectively are approximately constant, it can be concluded that this is true not only of adenylic but also of all the other precursors of RNA. We therefore conclude that the synthesis of RNA under our conditions is taking place from a pool that is in equilib-

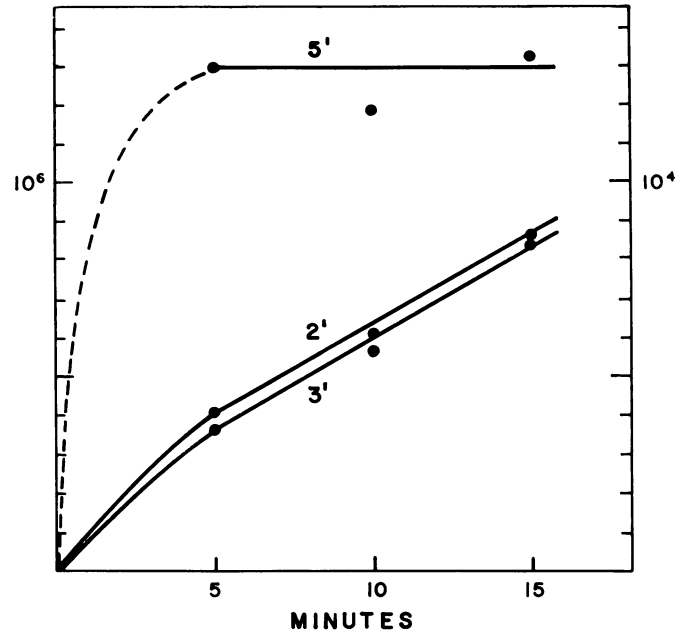


FIG. 7.—Specific activities of adenylic acids from yeast 59 RA growing in natural medium. Left ordinate—specific activity of 5′ isomer from pool. Right ordinate—specific activities of 2′ and 3′ isomers from RNA. (See text.)

rium with externally added isotope and that this pool is of constant specific activity.

(c) *Kinetics of uracil incorporation:* Under these conditions, the uptake of label into stable RNA should follow the equation $S = S_0 (e^{kt} - 1)$. If the size of the unstable fraction in Scheme 1 is zero, plotting ln $(S/S_0 + 1)$ against time should give a straight line passing through the origin. The value of S_0 can be estimated by plotting log (counts/min) and extrapolating the straight line portion of the curve on the ordinate (Fig. 4). We have measured the total uptake of uracil per milliliter of medium in exponentially growing cultures and obtained the results shown in Figure 8. Over a period of 11 hr, the plot of ln $(S/S_0 + 1)$ against time is not quite a straight line. Apparently, under our conditions growth is not strictly exponential, but the deviation is not very great (curve B). The same plot for three experiments for a shorter interval of 100–200 min is shown as curves A′, B′, and C in Figure 8. It will be seen that the rate is essentially linear from at least one min after addition of isotope (curve B′) but that the curves do not pass exactly through the origin. It can therefore be concluded that if there is an unstable fraction as required by Scheme 1, it is saturated in a period of less than a minute. Its size, as estimated from the intercepts of the curves on the ordinate, is 11, 2, and 4 per cent of total RNA for curves A′, C, and B′ respectively.

We believe, however, that failure of the lines to pass through the origin is an artifact mainly due to a systematic underestimate of S_0. One would expect that S_0 would in general be underestimated, since the plot of log (counts/min) against time will be strictly a straight line only when the value of S/S_0 is infinitely large

compared to 1: i.e., only many generation times after addition of isotope. At other times, its slope will be greater and therefore the intercept on the ordinate will be less than the true value of S_0. That this is indeed the case is demonstrated by Figure 9. For very small values of S, $\ln(S/S_0 + 1)$ is virtually proportional to S. We have therefore plotted the total counts from incorporated uracil against time for short periods of exposure to isotope. As can be seen from Figure 6, in six experiments the uptake is linear for the first 10 min of exposure and the line passes rigorously through the origin. Since the counts here are proportional to $\ln(S/S_0 + 1)$ but the plot is independent of any estimate of the value of

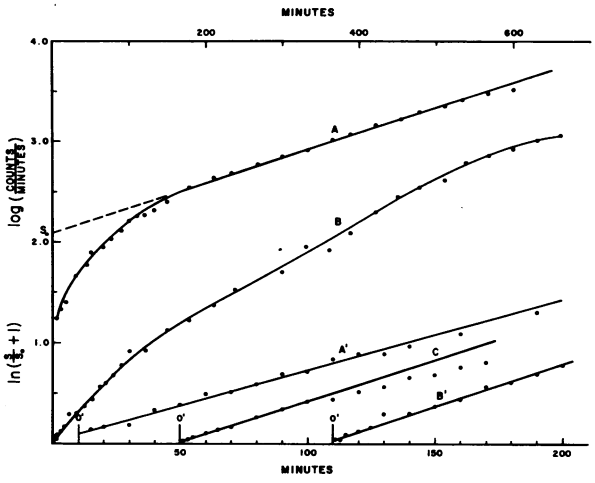


FIG. 8.—Uptake of uracil-C^{14} into yeast A-589B growing in synthetic medium. A—log (total counts). Other curves ln $(S/S_0 + 1)$. Upper time scale for curves A, B; lower for A′, B′, C′. Curves A′ and B′ are replotted from A and B respectively on different time scale.

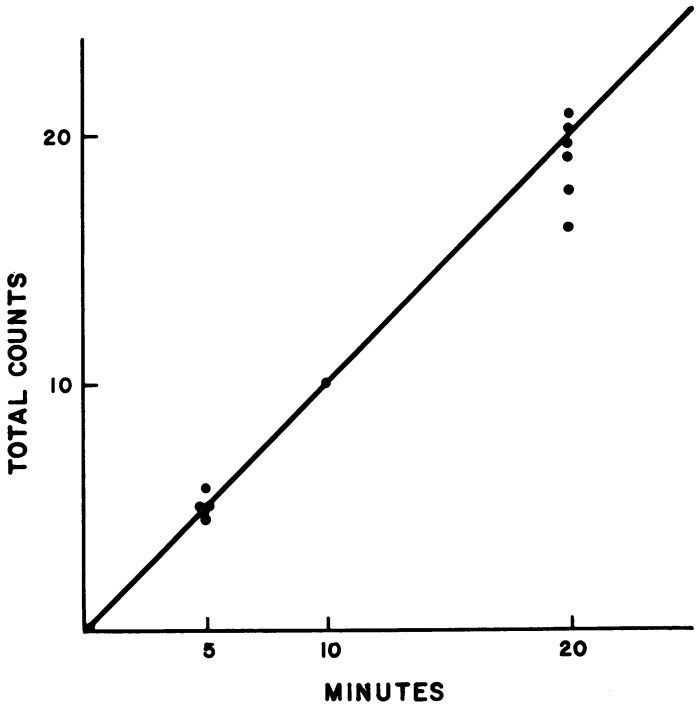


FIG. 9.—Uptake of uracil-C^{14} into cells of 59 RA growing in chemically defined medium. Total counts at 10 min normalized to 10.

S_0, this demonstrates that there is no fraction which is saturated in a few minutes. Our conclusion is that Scheme 2 best describes the flow of labeled material into RNA and that dRNA functions as an obligatory precursor of general cell RNA in the sense that all material which is incorporated into RNA must pass through this fraction.

(d) *Kinetics of P^{32} incorporation:* We have also studied the kinetics of phosphorus uptake into RNA by cultures growing in synthetic medium. The results are presented in Table 5. So far as the distribution of counts in individual 3′ nucleotides is concerned, up to a time of 15 min the composition of newly synthesized RNA approximates dRNA (experiments A and B), but at 30 and 60 min it is close to that of the bulk of the RNA (experiments C and D). It will be noted that at 5 min the calculated composition deviates widely from the other results. While we have experienced no difficulty in reproducibly obtaining a composition typical of dRNA after incubation in buffer, with growing cultures we have often obtained what appear to be aberrant calculated compositions after *short* exposures to P^{32}. Whether this is due to contamination of our preparations by some phosphorus-containing material which rapidly reaches a very high specific activity, or whether this indicates the existence of still another RNA fraction which becomes labeled more rapidly than dRNA in growing cultures is now being investigated.

In these experiments, the specific activity of the phosphate in the medium is known, so that if we assume that the specific activity of the internal phosphorus pool is the same as that in the medium, it is possible to calculate the actual amount of RNA synthesized and thus plot $\ln(S/S_0 + 1)$ against time as was done for the

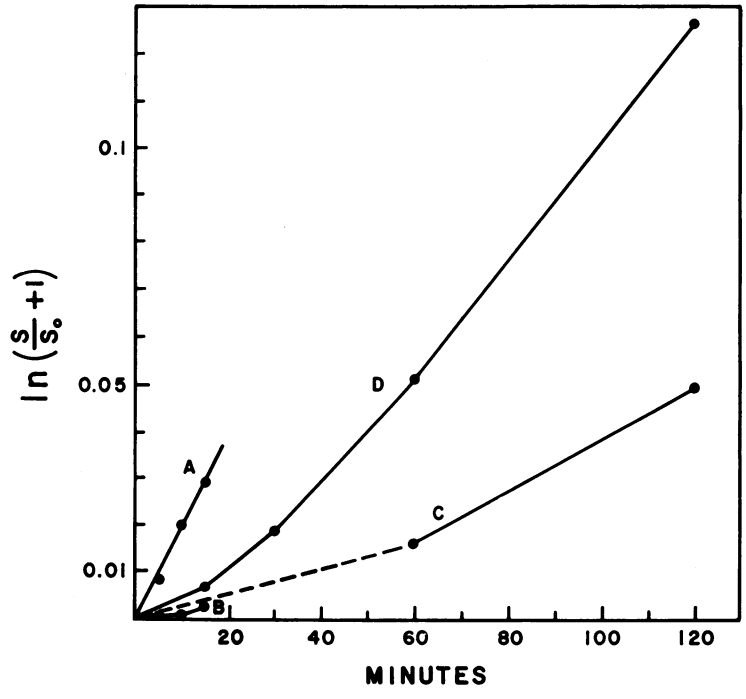


FIG. 10.—Plot of uptake of P^{32} from Table 5, as ln $(S/S_0 + 1)$ versus time. (See text.)

incorporation of uracil. Such a plot is shown in Figure 10. It will be noted that the initial rate of synthesis shows a lag and that the rates vary widely. We find that this is due to a variable use of internal phosphorus by yeast. In our synthetic medium, yeast will achieve almost maximum growth with 50 mg of KH_2PO_4 per liter. In Figure 11, we have plotted the rate of growth and uptake of phosphorus from the medium. It is evident that the initial uptake of phosphorus is in excess of that needed to sustain a maximum rate of growth since after virtually all phosphorus has been removed from the medium, growth, as measured by an increase in optical density, still continues at a rate almost identical to that of a parallel culture containing an excess of phosphorus. Evidently, the ratio of internal to external phosphorus used for synthesis varies with the state of the cells and conditions of the medium and it is thus not possible to obtain kinetic data directly with phosphorus in the same way as with uracil.

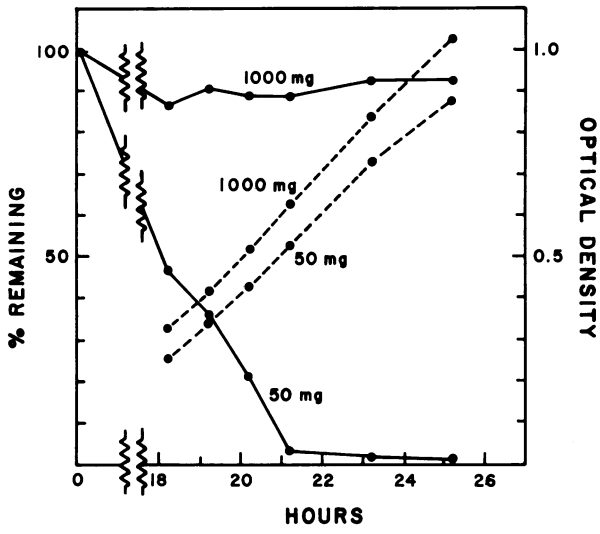


FIG. 11.—Uptake of phosphate by yeast 59 RA growing in synthetic medium with 1,000 mg and 50 mg/l of KH_2PO_4. Solid lines: P remaining in filtered medium, as determined from radioactivity. Dashed lines: optical density of cultures.

Discussion.—The results we present here show that during exponential growth added label enters the dRNA fraction at a rate which is equal to the rate of growth. Since the acid-soluble precursor pool maintains a constant specific activity, this is possible only if dRNA is a precursor of other stable RNA fractions. The conversion must occur without return of material from dRNA into the acid-soluble precursor pool. Stable RNA is mainly, of course, the RNA of ribosomes, so that dRNA can be considered to be a carrier of precursor nucleotides into ribosomal RNA.

Such a conclusion could have been reached on the basis of earlier findings by Halverson.[11] By studying the rate of disappearance of label from the acid-soluble precursor pool, he showed that in exponentially growing yeast the rate of nucleic acid breakdown is less than 0.037 per cent of the rate of synthesis, a completely negligible figure. If dRNA were a fraction in rapid equilibrium with the acid-soluble precursor pool, the results of Yčas and Vincent,[1] as well as those presented here indicate that it would form a very substantial fraction of the total RNA, and the rate of entry of isotope into dRNA would exceed by a considerable margin the rate of net RNA synthesis. Our findings that dRNA does not equilibrate with the acid-soluble precursor pool but rather is a polynucleotide precursor of ribosomal RNA satisfactorily reconcile these apparently contradictory results.

The accumulation of dRNA by yeast suspended in buffer presumably occurs because conversion to ribosomal RNA is not possible in the absence of protein syn-

thesis. Similar accumulation of dRNA has been recently reported to occur in bacteria when their rate of growth is decreased by "stepping down" into a less rich medium.[12]

We have no information on the mechanism by which dRNA is converted into ribosomal RNA. Since a change in composition is involved, it is evident that the net result must be a subtraction of nucleotides from dRNA. If nucleotides were added to dRNA, dRNA could not be detected by the type of experiments reported here.

In reporting the existence of dRNA, Volkin, Astrachan, and Countryman[3] and also Yčas and Vincent[1] speculated that it might function as a means of transmitting information from the gene, assumed to be DNA, to protein. This was based entirely on the finding that the composition of dRNA resembles that of the corresponding DNA. This speculation has since been repeated by a number of other workers (refs. 12, 13, 14). Our finding that dRNA is a precursor of ribosomal RNA lends no particular support to such speculations. As Yčas and Vincent[16] have already shown that bulk RNA from a single organism does not differ significantly in composition even when proteins of radically different composition are being synthesized, it is unlikely that ribosomal RNA is the bearer of the major part of the information determining the amino acid sequences of proteins (see also ref. 13). It is therefore also not likely that dRNA, functioning as a precursor of ribosomal RNA, is transferring much information into ribosomal RNA. This indeed follows from the fact that there is no clear correlation between the compositions of DNA (and therefore dRNA) and ribosomal RNA.

If the above is correct, one is faced with the problem of how the structure of ribosomal RNA is determined. We suggest the following: Normal cells have the capacity to produce an identical rather than a complementary copy of an RNA molecule; ribosomal RNA is produced by copying preexisting ribosomal RNA. This suggestion makes it possible to reconcile two apparently contradictory facts. On the one hand, all RNA appears to be of nuclear origin[17]; on the other, infection by viral RNA activates a copying process which appears not to exist in the normal cell. Our proposal is that the initial polymerization of nucleotides is indeed an exclusively nuclear event, involving a DNA template, but that the normal cell possesses the capacity to rearrange the nucleotides of the precursor dRNA into other sequences which are copies of RNA molecules already in existence in the cell. Such a mechanism does not necessarily imply that reproduction is either exclusively nuclear or cytoplasmic. If synthesis of ribosomal RNA occurs in this manner, then viral RNA replication is only a special case of a normally existing process.

Although, as we state above, our results do not indicate that dRNA is the bearer of genetic information, they also do not exclude the possibility that dRNA could act as such prior to its conversion into ribosomal RNA. Some of the indirect grounds for identifying dRNA with a "messenger" are genetic results which indicate that the "messenger" is unstable, as is dRNA itself,[18] and it has been postulated that this instability of dRNA is in some way the result of destruction of the "messenger" in the process of synthesizing protein.[13] Recently, however, evidence has accumulated that synthesis of proteins *in vitro* appears to involve a stable "messenger."[19] Such systems normally use cells and preparations in which no ribosomal synthesis appears to occur. Here again, our results make it possible to reconcile

the apparent contradiction between the stable and unstable "messengers." If dRNA is a "messenger," it is not destroyed by the act of protein synthesis *per se* but disappears by conversion into ribosomal RNA. It will therefore be unstable if synthesis of ribosomes is taking place, as in the growing yeast cultures described above, but it will be stable in systems where ribosomal synthesis is minimal, such as in yeast cultures in buffer and in reticulocytes synthesizing hemoglobin.

We stress, however, that on the basis of present evidence identification of dRNA with genetic "messengers" can be regarded only as provisional.

Summary.—We present evidence that in the absence of growth a fraction of RNA which resembles DNA in composition accumulates in yeast. This fraction appears to be an obligatory precursor of other molecular species of RNA present in the cell. The conversion of dRNA occurs without breakdown to material which reenters the acid-soluble precursor pool.

Note added in proof.—Two recent investigations which support our conclusion that dRNA is a precursor of ribosomal RNA have come to our attention. R. J. Britten, B. J. McCarthy, and R. B. Roberts have evidence that the rapidly labeled fraction of *E. coli* is a specific presursor of ribosomal RNA in this organism (*Biophysical J.*, in press). H. Chantrenne and S. Devruex have found that in the presence of 8-azaguanine synthesis of protein by *Bacillus cereus* is blocked, but synthesis of RNA continues *without much change in rate* (*Biochim. Biophys. Acta*, **39**, 486, 1960). The composition of the RNA synthesized in the presence of 8-azaguanine differs, however, from the composition of bulk RNA and approximates that of the DNA of *Bacillus cereus* (H. Chantrenne, *Arch. Intern. Physiol. et Biochem.* **69**, 745 (1961)).

We extend our thanks to Kenneth Paigen and Bernard S. Strauss for valuable discussions of these problems.

* The work reported here was supported by grants G-9753 from the National Science Foundation and C-3509 from the U.S. Public Health Service.

† On leave from the Biological Institute, Faculty of Science, University of Kobe, Japan.

‡ Senior Research Fellow, U.S. Public Health Service. Present address: Department of Anatomy, School of Medicine, The University of Pittsburgh, Pittsburgh 13, Pennsylvania.

[1] Yčas, M., and W. S. Vincent, these PROCEEDINGS, **46**, 804 (1960).

[2] The following abbreviations are here used: RNA—Ribonucleic acid; DNA—Deoxyribonucleic acid; Ad, Ur, Gu, and Cy—adenylic, uridylic, guanylic, and cytidylic acids.

[3] Volkin, E., L. Astrachan, and J. L. Countryman, *Virology*, **6**, 545 (1958).

[4] Cohn, W. E., and J. X. Khym, in *Biochemical Preparations*, ed. D. Shemin (New York: Academic Press, 1955), vol. 5, p. 40.

[5] Smith, J. D. in *The Nucleic Acids*, ed. E. Chargaff and J. N. Davidson (New York: Academic Press, 1955), vol. 1, p. 267.

[6] Filter Type HA Disc diameter 25 mm, pore size 0.45μ, obtained from the Millipore Filter Corporation, Bedford, Mass.

[7] Preiss, J., P. Berg, E. J. Offengand, F. H. Bergman, and M. Dickmann, these PROCEEDINGS, **45**, 319 (1959).

[8] Yčas, M., and G. Brawerman, *Arch. Biochim. Biophys.*, **68**, 112 (1957).

[9] Halverson, H. O., and S. Spiegelman, *J. Bact.*, **65**, 496 (1953).

[10] Takada, H., and S. Tokuno, *J. Inst Polytechnics*, Osaka City University Series D, **9**, 27 (1958).

[11] Halverson, H., *Biochim. Biophys. Acta*, **27**, 267 (1958).

[12] Hayashi, M., and S. Spiegelman, these PROCEEDINGS, **47**, 1564 (1961).

[13] Brenner, S., F. Jacob, and M. Meselson, *Nature*, **190**, 576 (1961).

[14] Gros, F., H. Hiatt, W. Gilbert, C. G. Kurland, R. W. Risebrough, and J. D. Watson, *Nature*, **190**, 581 (1961).

[15] Mean of results of Vischer, E., S. Zamenhof, and E. Chargaff, *J. Biol. Chem.* **177**, 429 (1949) and of Zamenhof, S., and E. Chargaff, *J. Biol. Chem.*, **187**, 1 (1950).

[16] Yčas, M., and W. S. Vincent, *Exp. Cell Research*, **21**, 513 (1960).
[17] Zalokar, M., *Exp. Cell Research*, **19**, 559 (1960).
[18] Jacob, F., and J. Monod, *J. Mol. Biol.*, **3**, 318 (1961).
[19] Bishop, J., G. Favelukes, R. Schweet, and E. Russell, *Nature*, **191**, 1365 (1961).

---

*SYNTHETIC POLYNUCLEOTIDES AND THE AMINO ACID CODE, III**

BY PETER LENGYEL, JOSEPH F. SPEYER, CARLOS BASILIO,† AND SEVERO OCHOA

DEPARTMENT OF BIOCHEMISTRY, NEW YORK UNIVERSITY SCHOOL OF MEDICINE

Communicated December 20, 1961

Previous work[1, 2] on the incorporation of amino acids into acid-insoluble products by a cell-free *Escherichia coli* system in the presence of synthetic polyribonucleotides led to the assignment of triplet nucleotide code letters (of as yet unknown sequence) for eleven amino acids. Since out of 64 triplets in RNA only six, corresponding to A + C + G permutations,[3] were not represented in the polymers tested, it seemed that code letters for more than eleven amino acids should be present among the 58 triplets covered by those polymers and that some of them might stimulate the incorporation of additional amino acids if it were possible to increase the over-all activity of the system.

It may be remembered that, of the various polymers tried, poly U accounted for one amino acid (phenylalanine), poly UC and UA for seven (isoleucine, leucine, lysine, proline, serine, threonine, and tyrosine), and poly UG and UAC for three (cysteine, histidine, and valine), and that poly UCG and UAG did not stimulate the incorporation of additional amino acids. Moreover, the activity of the system with poly UG, UCG, and UAG, as reflected by phenylalanine incorporation, was rather low. Accordingly, these polymers were selected for further study.

It has now been possible to boost the activity of the incorporation system with poly UG or poly UCG eight- to ten-fold by increasing the amount of (*a*) polymer, (*b*) transfer RNA, and (*c*) cold amino acids. At the same time, the sensitivity of the assay was augmented by increasing the specific radioactivity of the C^{14}-amino acid tested. In this way, three more amino acids, arginine, glycine, and tryptophan, have been added to the list. Their code letters are 1U 1C 1G, 1U 2G, and 1U 2G, respectively.

Preparations and Methods.—DL-asparagine-2,3-C^{14} was obtained from the New England Nuclear Corporation, Boston, Mass. All other preparations were as previously described.[1, 2] Poly UG (5:1) and poly UCG (6:1:1) were from the same batch of polymers used in the experiments of the preceding paper.[2] The experimental procedure followed closely that already described[1] except that the mixture containing ribosomes and supernatant (each with 5 mg protein) was preincubated for 30 instead of 15 min at 37° with no cold amino acids present and that the final reaction mixture contained transfer RNA in saturating amounts (1.3 to 2.5 mg, as assayed for phenylalanine incorporation in the presence of poly U);