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Ribosomal RNA Synthesis in Newly Sliced Discs of Potato Tuber

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Abstract. A burst of ribosomal RNA synthesis is induced in potato tissue by slicing, and continues at a decreasing rate for about 12 hours. Ribosomal RNA synthesis in potato discs is sensitive to puromycin, in contrast to non-ribosomal RNA synthesis. Thus, the influence of puromycin on total RNA synthesis is significant only during the first 12 hours following slicing. The function of RNA made after 12 hours in a puromycin-insensitive manner is unknown. However, it is apparently unrelated to protein synthesis, since it has been shown that total inhibition of RNA synthesis by addition of actinomycin D to potato tissue after 12 hours of aging has no effect upon protein synthesis during the ensuing 12 hours.

Click and Hackett (4) have shown that the synthesis both of RNA and of protein is essential to the time-dependent rise in respiration that takes place when the potato tuber is cut into thin (1 mm) slices. Figure 1 shows data presented by them in their figure 3 (4) replotted to show clearly the effects of puromycin and of actinomycin D on both RNA and protein synthesis as a function of the time of addition of inhibitor. The effect of actinomycin on leucine incorporation (A/L), and of puromycin on uracil incorporation (P/U), which we call "reciprocal" inhibition, is manifested mainly in the first 8 to 12 hours following slicing. While reciprocal inhibition in both cases ends approximately 12 hours after slicing, "direct" inhibition, *i.e.* the effect of actinomycin on uracil incorporation (A/U), and of puromycin on leucine incorporation (P/L), is evident throughout the 24 hours of the experiment. The actual decrease in the sensitivity of protein synthesis to puromycin over the first 12 hours (Click, personal communication) is approximately but 6 % of the decrease in puromycin sensitivity of RNA synthesis noted in our experiments during the same interval.

The observations suggest that the synthesis of an RNA whose formation is puromycin sensitive is more or less complete in 12 hours, while puromycin-insensitive RNA synthesis subsequently continues. The noted reciprocal relationship and its time course can be explained by making 2 assumptions: First, that slicing initiates ribosome formation (involving synthesis of RNA and protein components) which continues at a decreasing rate during the ensuing 12 hours, and secondly that RNA formed in the absence of protein synthesis is unstable, as is the case in bacterial systems (6).

Evidence has been presented that where RNA synthesis is inhibited by puromycin aminonucleoside, an inhibitor of protein synthesis more sensitive than puromycin, it is ribosomal RNA synthesis which is preferentially suppressed (7, 8). We have investigated RNA synthesis in potato discs to determine whether there is an initial synthesis of ribosomal RNA which falls to a low level over the 12 hours following slicing. It is of particular interest in connection with this study that puromycin inhibits the respiratory rise which develops over 24 hours in potato slices, only when added in the first 12 hours (4, 12). As a matter of convenience we will frequently speak of puromycin sensitive or insensitive RNA, when in fact we are referring to the sensitivity of RNA synthesis.

Materials and Methods

Russet-Burbank potato tubers were purchased locally. Discs 1.0 mm thick and 9.0 mm in diameter were prepared in a standard way (11) and preincubated for a stated period in a solution containing phosphate buffer (0.02 M, pH 6.7), $MgCl_2$ (5×10^{-3} M), $CaSO_4$ (5×10^{-4} M), dihydrogen streptomycin sulfate (50 μg/ml) and Na methicillin (0.5 mg/ml). After the stated preincubation periods in the absence of radioisotope, batches of discs were exposed to uracil-2-^{14}C. The total uptake of isotope into whole discs, and the incorporation into hot alcohol-insoluble material, was determined as previously described (12).

Preparation of RNA. The method of Click and Hackett for RNA isolation and characterization (5) was used in a slightly modified form. The discs (10 g) were ground in 20 ml of 1 % sodium lauryl

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sulfate in glycine buffer (pH 9.1), plus 40 ml of phenol saturated with water containing EDTA (10^{-3} M, pH 7.0). The homogenate was then shaken at 55° for 15 minutes, a procedure found to give a greater yield of RNA than extraction at room temperature. Further purification and sucrose gradient fractionation were as described by Click and Hackett (5).

Bacterial Contamination. To reduce bacterial contamination, streptomycin sulfate (50 μg/ml) in conjunction with frequent changes of incubation solution has been used (12). After 24 hours of incubation at 22 to 23° with streptomycin sulfate under the standard conditions, discs yielded 2.5×10^6 bacteria/g fresh weight when the total homogenate, prepared by grinding discs in 1 % NaCl, was plated onto nutrient agar. By the further addition of Na methicillin (0.5 mg/ml) to the incubation medium, bacteria were reduced to less than 4 % of this number. The 2 antibiotics showed no significant effect upon either the uptake or the incorporation of uracil-2-^{14}C (table I), or upon the respiratory

Table I. *The Effect of Antibiotics on Uptake and Incorporation of Uracil-2-^{14}C by Discs of Potato Tuber*

Antibiotic concentration: methicillin (0.5 mg/ml), dihydrogen streptomycin sulfate (50 μg/ml). Discs were incubated in a standard way (11) in medium containing uracil-2-^{14}C (30 mc/mmole, 0.3 μc/ml) for 4 hours before being extracted with hot ethanol.

	Uptake	Incorporation
	dpm/g fr wt	
+ Antibiotics	2.38×10^5	5640
— Antibiotics	2.56×10^5	6250

increase evinced by potato discs. Streptomycin does however stop volume growth in potato discs (15). Since as many as 5×10^6 bacteria/g fresh weight were shown to have no effect upon adenine incorporation in excised pea segments (16), and since the potent bacteriostat, puromycin, has little effect upon RNA synthesis when added after 12 hours of aging (fig 1, table II), contamination can have no effect upon the results to be described.

Actinomycin D. Solutions of actinomycin D were made up and used in flasks protected from light. Without this precaution the inhibition of uracil incorporation in discs pre-aged for 6 hours or more was reduced and erratic.

Results and Discussion

To determine whether the synthesis of a component of potato RNA is susceptible to puromycin inhibition, and whether the synthesis of this component becomes negligible some 12 hours after slicing, batches of discs were preincubated (aged) for 1 and one-half, 6, 10, or 18 and one-half hours

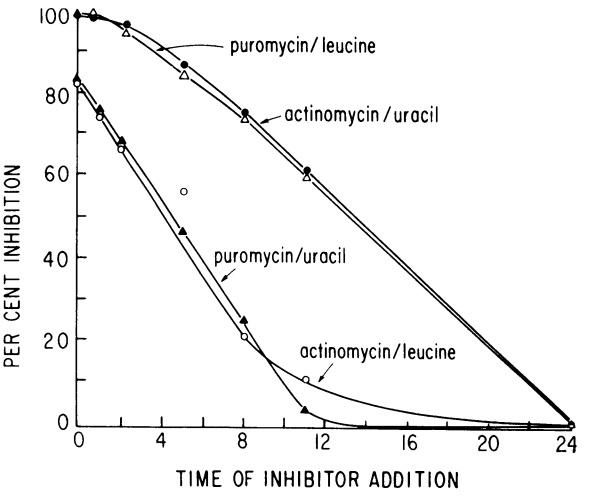

FIG. 1. The effects of delayed additions of puromycin and actinomycin D on the incorporation of uracil-2-^{14}C and leucine-1-^{14}C respectively. Puromycin and actinomycin to final concentrations of 5×10^{-4} M and 25 μg/ml, respectively. The inhibitors were added after 0, 1, 2 and one-half, 5, 8, or 11 hours of incubation, and all incorporation measurements were made at the end of 24 hours. Uracil-2-^{14}C was present throughout the full 24 hours. [Replotted from Click and Hackett (4, fig 3)].

before incubation for 4 hours in uracil-2-^{14}C. Following the exposure to labeled uracil, the discs were rinsed and then extracted with hot alcohol (12) to determine both total uptake and incorporation of uracil. It was separately determined that actinomycin D (50 μg/ml) inhibits uracil incorporation by at least 95 % in any interval during 24 hours. Thus, the noted incorporation reflects DNA-directed RNA synthesis (10). To determine the puromycin sensitivity of the RNA synthesis, puromycin (5×10^{-4} M) was added to one-half of each batch of discs 90 minutes before exposure to isotope. The results of the experiments are given in table II. Two points are clear: first, that puromycin sensitive RNA synthesis is markedly reduced in the first hours, and secondly, that total RNA synthesis is more or less constant in any 4 hour period. That is to say, the

Table II. *The Effect of Puromycin on Uracil Incorporation into Potato Disc RNA*

Batches of discs were aged in non-radioactive medium for 1 and one-half, 6, 10, and 18 and one-half hours respectively before the addition of 2-^{14}C uracil (30 mc/mmole, 0.6 μc/ml, 25 ml/5 g discs) for 4 hours. 90 minutes before the addition of isotope, puromycin (5×10^{-4} M) was added to one-half of each batch.

Hr preincubation	Uracil-2-^{14}C incorporation into RNA of whole discs: Control	Puromycin	Inhibition by puromycin
	dpm per sample		%
1.5	10,100	6,930	31
6.0	9070	7800	14
10.0	9675	8930	8
18.5	10,800	10,180	6

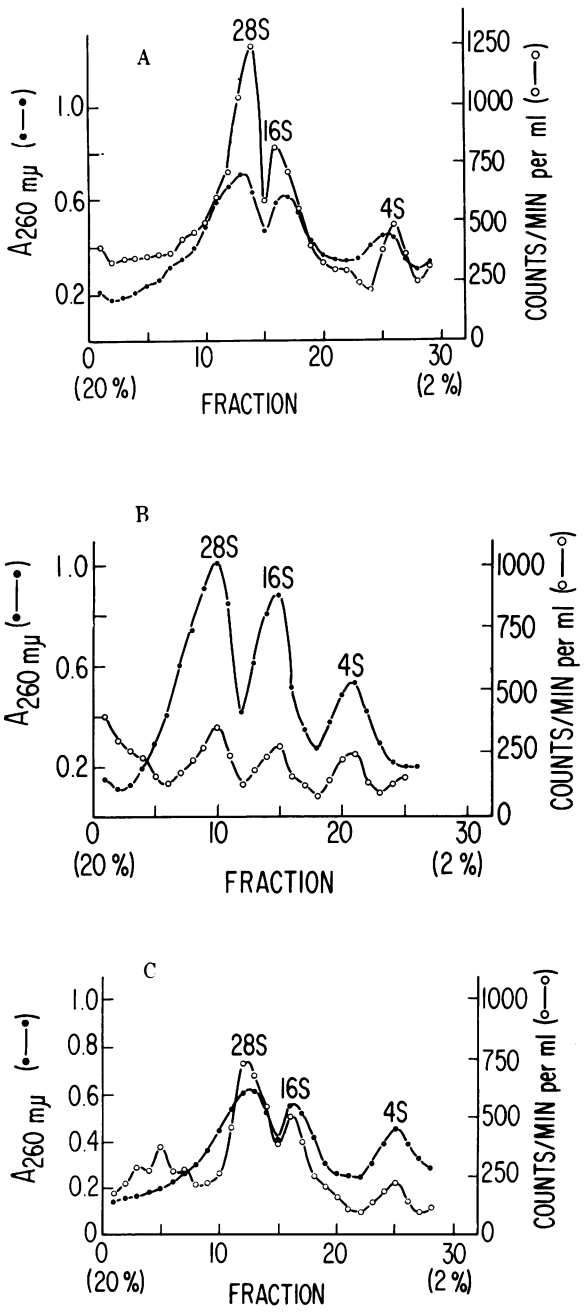

FIG. 2. Sucrose density gradient fractionation of 2-^{14}C-uracil labeled RNA of potato discs. A) Discs labeled from 0 to 4 hours. B) Discs labeled from 0 to 4 hours + puromycin (5×10^{-4} M). C) Discs labeled from 12 to 16 hours. One-half of the phenol extracted RNA (1 mg) from 10 g lots of discs labeled for 4 hours with uracil-2-^{14}C (52.6 mc/mM, 12.5 μc/25 ml/10 g discs) was layered onto a 20 ml sucrose gradient (2–20 % w/w) (5) and centrifuged for 16 hours at 24,000 rpm in a Spinco SW 25.1 Rotor. One ml fractions were collected by upward displacement of fractions through a fine tube extending to the bottom of the gradient. Hence the "early" fractions contain the heaviest components, and represent the densest part of the gradient. Samples were diluted with water (1 ml) and the optical density at 260 mμ measured. Fractions were precipitated with equal volumes of 10 % TCA in 10 % acetone, using serum albumin carrier, and were collected on glass filter papers. The precipitate was washed with 5 % TCA and 95 % ethanol, air dried and counted in a liquid scintillation counter.

proportion of the total RNA synthesis that is insensitive to puromycin increases with aging from 70 % initially, to more than 90 % in the latter 4-hour periods.

Figure 2 shows the results of an experiment in which freshly sliced discs were incubated in uracil-2-^{14}C for 4 hours either with (fig 2B) or without (fig 2A) puromycin. Figure 2C shows the result of preincubating the discs for 12 hours before the addition of labeled uracil. RNA was phenol extracted and run on a sucrose gradient as indicated. Ribosomal RNA accounts for 80 % of the recovered RNA (OD 260 mμ), and most of the recovered radioactivity in each case. That there is not complete coincidence between OD values and radioactivity, particularly with respect to RNA sedimenting more rapidly than 28 S ribosomal RNA, points to the presence of a heavier RNA species. In this connection it is to be noted that nuclear RNA of HeLa cells is both heavy, and extremely polydisperse, when isolated under conditions similar to ours (1). Our peaks would appear to sit on a base of this polydisperse material (*vide infra*).

It is to be noted that the puromycin insensitive RNA synthesis which rises during aging from 70 % to more than 90 % of the total RNA labeled in a 4 hour period is not accounted for in the phenol extracted RNA. This puromycin insensitive RNA is largely lost during phenol extraction, and does not appear as a discrete RNA species on the sucrose gradient. It is probable that some of it, however, becomes smeared across the gradient to yield the basal level alluded to above. This contaminating RNA exerts a larger distorting effect on the true picture of uracil incorporation into ribosomal RNA when the rate of ribosomal synthesis is low (fig 2C) than when it is high (fig 2A), thus accounting for the fact that the specific activity of the ribosomal RNA synthesized from t_{12} to t_{16} does not decrease to the extent predicted by table II from the level of puromycin sensitivity. Nevertheless, figure 2 indicates that puromycin sensitive RNA synthesis is predominantly ribosomal, and ribosome synthesis decreases sharply with aging. While the reduction with aging in figure 2 is less than that indicated in table II, as deduced from puromycin sensitivity, the diminution of ribosomal RNA synthesis with aging, as determined in figure 2, may range from 50 to 70 % from one experiment to another. Puromycin reduces the measured amount of ribosomal RNA synthesis in fresh discs by some 70 % (fig 2A, B). Aging *per se* for 12 hours leads to more than a 50 % reduction in ribosomal RNA synthesis in a subsequent 4 hour period.

Puromycin insensitive RNA is found primarily in the pellet derived from relatively low speed centrifugation, and has been isolated by a Schmidt-Thannhauser extraction from a pellet sedimented at 10,000 × *g* (10 min). The Schmidt-Thannhauser extraction avoids the loss of low molecular weight RNA components which unavoidably attends the alcohol precipitation steps of phenol extraction. For reasons which will become apparent, we have limited tissue fractionation to supernatant and particulate fractions, the latter referring to all components which are sedimented at relatively low centrifugal forces (10,000–17,000 × *g*). Evidence will be presented (table III) to show that this RNA, whose synthesis is puromycin insensitive, and can be blocked with actinomycin D without effect on protein synthesis (fig 1, A/L, 12–24 hr), is metabolically labile. It is very tentatively suggested that this RNA represents "nuclear RNA" which has been described as metabolically labile and unrelated to protein synthesis (9, 13). Further, in HeLa cells the "nuclear RNA" is puromycin aminonucleoside insensitive in contrast to the ribosomal RNA (7, 8 *cf.* 1).

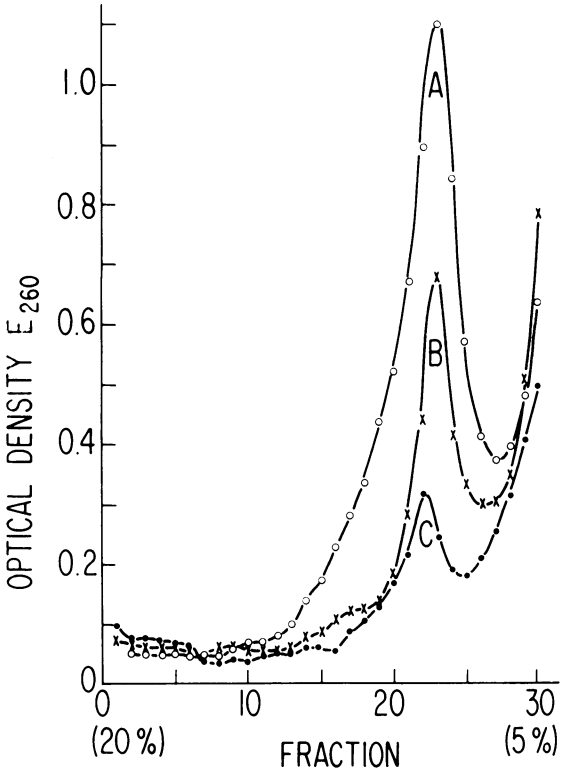

FIG. 3. Sucrose density gradient fractionation of ribosomes of fresh and aged potato discs. A) Fresh discs extracted in NP40. B) 5 hour aged discs extracted in NP40. C) 5 hour aged discs extracted without NP40. Tissue (5 g) was ground in a mortar at 0° in a medium (5 ml) containing tris buffer (0.05 M pH 7.8), magnesium acetate (4 × 10^{-3} M) and Cleland's reagent (5 × 10^{-3} M) (3). Where present, the non-ionic detergent Nonidet P40 (NP40, Shell Chemicals) was used at a concentration of 0.5 % v/v. The homogenate was centrifuged for 10 minutes at 12,000 × *g*, and 1.0 ml of the supernatant was layered onto a linear sucrose gradient (29 ml, 5–20 % in 0.05 M tris pH 7.4, magnesium acetate 5 × 10^{-3} M). Centrifugation was for 2 hours at 24,000 rpm in a Spinco SW 25.1 rotor.

Initial determinations of ribosome prevalence both by sucrose density gradient analysis and by analytical centrifugation pointed to a decrease in the total ribosome content with aging over and above the decrease in ribosome synthesis with time. It should be noted the vast bulk of potato ribosomes were synthesized the previous year during tuber formation. The net disappearance of ribosomes is artifactual, arising from the adherence of ribosomes to the membranous fraction of the homogenate, which increases markedly with slice aging. Figure 3 shows the decrease in the recovery of ribosomes from a given quantity of tissue with aging, even with the nonionic detergent Nonidet P40 (NP40, Shell Chemicals, 0.5 % v/v) in the homogenizing medium. Triton X-100 (1 % v/v) yielded fewer ribosomes than did NP40. Deoxycholate was similarly ineffective, although the ineffectiveness could have resulted from precipitation of deoxycholate with Mg^{2+}, discs being aged in $MgCl_2$ (5 × 10^{-3} M). As the direct extraction of ribosomal RNA (OD_{260}) yields equal quantities of RNA from equal fresh weights (3.5 g) of fresh and aged discs, an examination was made of the RNA centrifuged down at 17,000 × *g* in the presence of Triton X-100 (1 % v/v). Figure 4 shows the sucrose density gradient pattern of phenol extracted RNA from both the pellet and the supernatant of this low speed centrifugation, for fresh and for 5 hour aged discs. There is a substantially increased loss of ribosomal RNA to the 17,000 × *g* pellet in the aged discs. We have been unable to increase the yield of free ribosomes by a brief RNAse treatment, and feel that it is highly unlikely that the rapid sedimentation of ribosomes reflects polyribosome formation. More probably there is a non-specific trapping of ribosomes by membranes, so that both cell fractionation and polyribosome studies are likely to be misleading. This is in keeping with the cell-free protein synthesis studies of Chapman and Edelman (2) who found that the protein incorporation system in Jerusalem artichoke, presumably ribosomes, was chiefly in a fraction sedimenting at 15,000 × *g*. Their 105,000 × *g* fraction contained only 10 to 30 % of the activity of the low speed sediment. We have, therefore, preferred to measure ribosome synthesis indirectly by determining ribosomal RNA, and do not specify the location of the puromycin insensitive RNA beyond saying that it is particulate.

While the decrease in puromycin sensitivity of RNA synthesis (uracil incorporation) with time displayed in figure 1 is explicable in terms of a decrease in ribosome synthesis, there are 2 anomalies which call for explanation. First, while actinomycin is just as effective when added after 3 hours as when added initially in inhibiting uracil incorporation

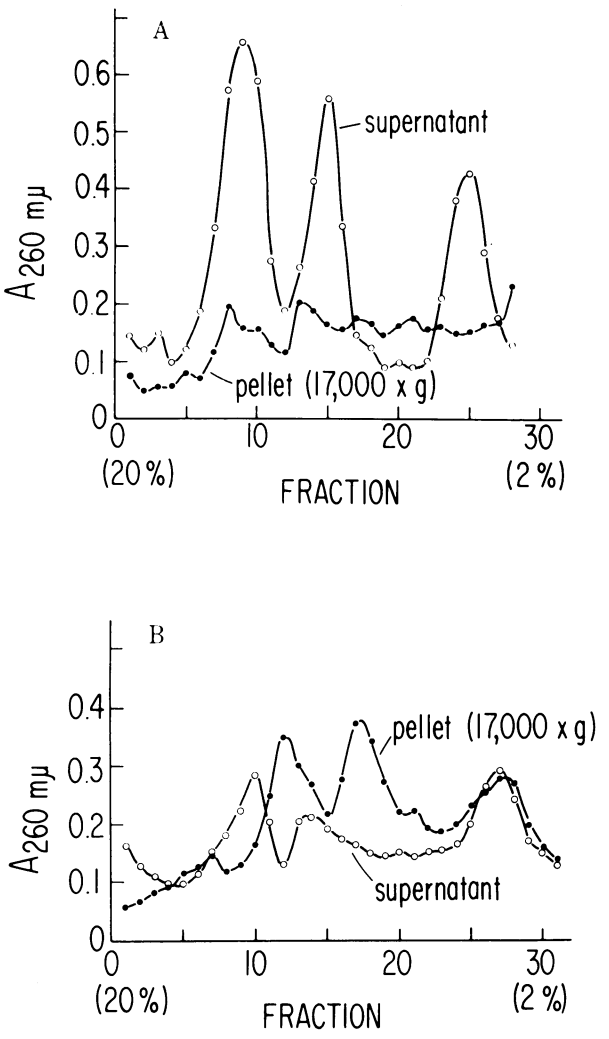

FIG. 4. The distribution of RNA between the pellet and supernatant of a centrifuged potato homogenate. Homogenization in Triton X-100 (1% v/v) followed by centrifugation at 17,000 × *g* for 10 minutes. RNA phenol extracted as described in Methods. Density gradient centrifugation as indicated in figure 2. A) Fresh tissue. B) Discs aged 5 hours before extraction. When ribosomes are first pelleted at high speed (105,000 × *g*, 3 hr) subsequent phenol extraction yields a single, slowly sedimenting fraction with a broad peak, rather than the sharp peaks of ribosomal RNA which are derived from the direct phenol extraction of the homogenate. Even low speed centrifugation (17,000 × *g*, 10 min) leads to some alteration of the pellet-associated ribosomal RNA, possibly as the result of partial enzymatic degradation.

through a 24 hour period, puromycin is effective from t_0 in the inhibition of uracil incorporation in a 24 hour period (compare curve A/U with P/U in fig 1). That is, ribosome synthesis starts at once, as indicated by the reciprocal inhibition of uracil incorporation by puromycin, and it is not immediately evident why actinomycin appears to be without effect in the first 3 hours (remembering that the experimental period is a full 24 hours in all cases). Secondly, to the extent that the reciprocal inhibition curve (P/U) is steeper than the direct inhibition curve (A/U), RNA synthesis in the first 10 hours is greater when judged by its response to puromycin than when judged by its response to actinomycin. That is to say, there is more of a difference per unit time as a consequence of delaying puromycin presentation than of delaying actinomycin presentation. Since, however, actinomycin inhibits all RNA synthesis, including ribosomal RNA synthesis, the slope of the actinomycin curve should be greater than that for the puromycin curve. The data of Click and Hackett (4), presented in figure 1, and of Laties (12) shows the reverse. The apparent anomalies may be explained on the following basis. The puromycin insensitive RNA is metabolically labile, and its breakdown is thus a function of time. In a 24 hour incubation period where actinomycin is added after 4 hours, for example, the puromycin insensitive RNA synthesized before actinomycin addition, is destroyed in the 20 hours remaining before the end of the experimental period. Both because the synthesis of puromycin insensitive RNA increases with time (table II), and because the earliest formed unstable RNA has the greatest time in which subsequently to be degraded, the "apparent" influence of actinomycin increases with time. Since figure 1 represents a full 24 hour incubation period in all cases, early presentation of actinomycin results in the illusion of little actinomycin sensitivity in the period t_0–t_3. However, since actinomycin inhibits all RNA synthesis, ribosomal RNA included, some evidence of actinomycin inhibition should be manifest in the first 3 hours; that is, the slope of the actinomycin curve should be steeper than for the puromycin curve. For these reasons it is compelling to presume that in the absence of continued RNA synthesis there is a breakdown not only of puromycin insensitive RNA, but also of earlier formed puromycin sensitive ribosomal RNA which is stable under normal conditions.

Table III provides direct evidence for the existence of a metabolically labile RNA. Five batches of discs were exposed to uracil-2-^{14}C for 6 hours following slicing. At the end of the exposure period, the discs were washed with incubation medium containing ^{12}C uracil (0.2 mg/ml). One batch was then extracted (12), while the remaining 4 batches were incubated in medium containing ^{12}C uracil (0.2 mg/ml) for an additional 14 hours. To 1 batch was added puromycin, and to a second, cycloheximide (actidione) (14), both inhibitors of protein synthesis. Actinomycin D was added to a third, while the fourth batch was used as a control, being aged in the absence of inhibitors over the 14 hours following the removal of isotope.

The increase in incorporation that takes place after the removal of uracil-2-^{14}C (table III) shows that there is considerable subsequent utilization of isotope taken up during the previous period, as

Table III. *Incorporation of Previously Absorbed Uracil-2-^{14}C Into Potato Disc RNA*

Uracil-2-^{14}C (30 mc/mmole, 0.5 μc/ml) was presented for 6 hours following slicing. One sample was then extracted. The remaining 4 batches were incubated for a further 14 hours in nonradioactive medium containing uracil (200 μg/ml). Where present inhibitors were added at the following concentrations: puromycin (5×10^{-4} M), cycloheximide (actidione) (500 μg/ml) and actinomycin D (50 μg/ml). At 50 μg/ml a 97% inhibition of RNA synthesis has been obtained when actinomycin and uracil-2-^{14}C were continuously present from t_0 to t_{18}.

Duration and conditions of incubation		Uracil-2-^{14}C incorporation	Inhibition
0-6 hr	*6-20 hr*	*dpm*	%
Uracil-2-^{14}C	...	2780	...
"	Uracil (unlabeled)	10,300	...
"	Uracil + Puromycin	8830	14
"	Uracil + Cycloheximide	7760	25
"	Uracil + Actinomycin D	1610	84

observed by Laties (12). The experiment shows a limited and similar inhibition of uracil-2-^{14}C incorporation by puromycin and by cycloheximide in the period 6 to 20 hours following slicing. RNA synthesis sensitive to inhibitors of protein synthesis represents only a small fraction of the total RNA synthesis after 6 hours. Actinomycin, however, inhibits all further synthesis of RNA.

Significantly, the level of radioactivity in the actinomycin treated discs after 20 hours, is 56% less than that found in discs extracted immediately on their removal from label, *i.e.*, between hours 6 and 20 there has been a loss of approximately half of the RNA formed during the 6 hours following slicing. This destruction of preformed RNA accounts in part for the apparently similar effectiveness of actinomycin when presented at t_0 or t_3 hours (fig 1). In addition, it should be noted that it is because of the labile nature of the puromycin insensitive RNA that total RNA synthesis over 24 hours is so sharply reduced (80%) when puromycin is added at t_0, (fig 1). If the puromycin insensitive RNA were not metabolically labile, then puromycin would reduce total RNA synthesis over 24 hours by only 15 to 20% (avg of puromycin sensitivity of RNA synthesis in consecutive 4 hr periods in table II), *i.e.*, over 24 hours the total synthesis of ribosomal RNA reflects the steady state condition in which about 75% of the total RNA, as measured chemically or spectrophotometrically, is ribosomal RNA.

Acknowledgments

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