exposure of the iris tissue to the same concentration of norepinephine in Tyrode's solution in the presence of ATP (again followed by four washings in Tyrode's solution) resulted in a marked increase of the fluorescence intensity of the adrenergic fibers as well as the density of the fluorescent fibers that were visible. Even at this reduced exposure, Fig. 2*c* is too bright to show clearly the microscopic picture. The photomicrograph in Fig. 3 was therefore taken from another iris preparation treated in the same manner as in Fig. 2*c* but a reduced photographic exposure was used. The latter photomicrograph shows clearly that the uptake of norepinephrine by the tissue in the presence of ATP is confined within nerve structures.

Direct chemical determinations (*8*) of norepinephrine were made in pooled tissues from 16 irises per group. Each pool weighed approximately 9 mg. Values for the control (as in Fig. 2*a*) and thoroughly washed preparations treated with norepinephrine plus ATP (as in Figs. 2*c* and 3) were 5.2 and 36.1 μg/g, respectively.

These results indicate that in the presence of ATP, exogenous norepinephrine is taken up within the adrenergic fibers. It is of interest that in this tissue ATP is essential for a significant uptake of norepinephrine—a finding which lends support to the view that ATP is necessary for the binding of norepinephrine within the adrenergic storage sites (*9*). The results confirm previous observations made with tritiated norepinephrine and autoradiography (*10*); however, under the present conditions an extensive net uptake of norepinephrine was demonstrated.

Apart from their physiological significance, the findings indicate that this method, in which the tissue is exposed to exogenous norepinephrine in the presence of ATP, can be used in conjunction with the formaldehyde condensation procedure to provide a more sensitive histochemical method for the demonstration of adrenergic fibers in tissues. With this modification the finer branches of the fibers become visible and in general the preparations can be studied and reproduced photographically with greater ease.

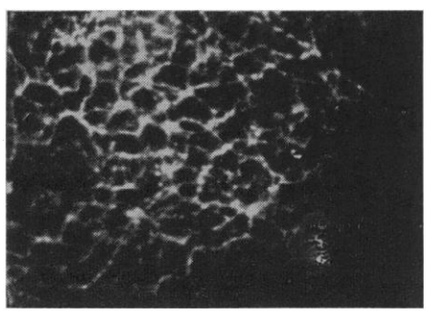

Fig. 3. A different iris preparation treated in the same way as that in Fig. 2*c* but photographed with 1-second exposure.

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20 April 1964

Hybridization of Rapidly Labeled Nuclear Ribonucleic Acids

Abstract. *RNA, prepared from strain L fibroblasts which were incubated for 30 minutes in radioactive cytidine, was examined by quantitative DNA-RNA hybridization tests, autoradiography, and sedimentation analysis. About 60 percent of the radioactive RNA consists of 38 to 45S molecules located in the nucleoli and capable, under certain circumstances, of forming hybrids with DNA. Unlabeled 28S and 18S ribosomal RNA can compete with these components for DNA sites, indicating that the conversion of nucleolar RNA to ribosomal RNA occurs in such a way that base sequences are preserved. The polydisperse components associated with the chromatin region contain base sequences different from ribosomal and 4S RNA, and consequently have a uniquely high specific activity. These components are the only detectable hybridizing species in unfractionated preparations of RNA.*

In most cells rapidly labeled RNA appears at two distinct sites: the nucleoli and the extranucleolar or chromatin portions of the nucleus. The RNA labeled in the nucleolar region consists of large 38 and 45*S* molecules, the nucleotides of which ultimately become part of the 18 and 28*S* ribosomal RNA (*1–4*). Kinetic studies and base composition analyses (*3, 4*) suggest that a direct conversion, 45*S* → 38*S* → ribosomal RNA, occurs. What remains to be shown is that the large precursor molecules actually contain reasonably long base sequences identical to those of ribosomal RNA.

Chromatin-RNA is made up of a 4*S* component and of polydisperse components sedimenting between 8*S* and 45*S* (*1, 2*). The polydisperse components, metabolically unstable in the absence of concomitant ribosomal RNA synthesis, were suspected of being messenger RNA's. Although rapidly labeled RNA complementary to DNA (*3, 5*) or having a DNA-like base composition (*6*) has been described for mammalian cells, a definite identification with the chromatin fraction has not been made.

In this report we seek to characterize further the various rapidly labeled RNA fractions by studying their ability to form specific hybrid molecules with DNA and by observing whether there is a competitive effect of ribosomal RNA on the hybridization. Such techniques made possible the detection of DNA sequences complementary to the various kinds of bacterial RNA (*7, 8*). To distinguish between the rapidly labeled nucleolar and chromatin fractions we make use of the fact that cells exposed to low concentrations of actinomycin D synthesize normal amounts of RNA in the chromatin region, but have a greatly reduced capacity to make nucleolar RNA (*9*).

Strain L fibroblasts grown in spinner culture were used as a source of RNA (*1*). Pulse-labeled RNA was obtained by phenol–sodium dodecylsulfate extraction (*1*) of cells incubated for 30 minutes at 37°C with 62.5 μc of cytidine-2-C^{14} per liter (20 mc/mmole) or with 500 μc of cytidine-H^3 per liter (2.52 c/mmole). Where indicated, actinomycin D (0.04 μg/ml) was added to the culture 10 minutes before the pulse of labeled cytidine.

For autoradiography 10-ml portions of the labeled cell suspensions were centrifuged. The pellets of cells, after one washing with medium containing 50 percent calf serum, were fixed in Carnoy's solution, rinsed in 70 percent ethanol, dehydrated in absolute alcohol, cleared in toluene, and embedded in paraffin. The solid pellets were cut into sections 2 μ thick, and autoradiographs were made by standard techniques (*10*).

The hybridization studies were carried out with the agar-gel technique of Bolton and McCarthy (*8*) in which heat-denatured DNA, entrapped in an agar gel, is allowed to hybridize with various RNA samples. The DNA, extracted from frozen pellets of L cells as described by Szybalska and Szybalski (*11*) and denatured by heating at 100°C for 10 minutes and quick chilling, was entrapped in Oxoid agar–agar No. 3. The appropriate samples of labeled RNA in 0.7 ml of double-strength SSC (SSC: 0.15*M* NaCl, 0.015*M* sodium citrate) were incubated at 60°C for 48 hours with 0.7 g of agar containing 250 μg of DNA. The unbound RNA was eluted from the agar with nine successive 15-ml washes of double strength SSC at 60°C. The temperature was then raised to 70°C, and the hybridized RNA was eluted with two 15-ml washes of SSC diluted 1 to 100. A further increase in the temperature to 75°C did not cause a significantly greater amount of RNA to be eluted from the agar. After the addition of 2.5 mg of serum protein as carrier, the RNA in the washes was precipitated at 3°C in 5 percent trichloroacetic acid and collected on Millipore filters. The radioactivity was assayed in a scintillation counter, and the percentage of hybrid-forming molecules was calculated as the ratio of the number of counts per minute eluted at 70°C to the total number of counts per minute eluted from the agar.

Typical sedimentation patterns of RNA's from cells that had been pulse-labeled with cytidine-C^{14}, with and without actinomycin treatment, are shown in Fig. 1. Whereas the RNA from control cells exhibits peaks of radioactivity at about 45*S* and 38*S*, some polydisperse material, and a peak at 4*S*, the radioactive RNA from cells treated with $3 \times 10^{-5}M$ actinomycin is polydisperse except for a 4*S* peak. In actinomycin-treated cells the amount of cytidine incorporated during a 30-minute pulse is about 44 to 54 percent of that observed in control cells; the specific activity (counts per minute per optical density unit) of the 4*S* peak ranges from about 70 to 130 percent of the control.

Table 1. Hybridization of pulse-labeled RNA from normal and actinomycin-treated cells.

RNA incubated with DNA-agar* (μg)	Radioactivity (count/min)		Labeled RNA bound [(*B*/*A*) × 100] (%)
	Total recovered from agar (*A*)	Eluted at 70° to 75°C (*B*)	
	Normal cells†		
10	878	83	9.4
18	1515	125	8.2
31	3163	256	8.1
	Actinomycin-treated cells‡		
23	751	131	17.4
41	1348	209	15.5

* 250 μg of DNA. † 400 ml of cells (3.8×10^5 cells per milliliter) grown for 30 minutes at 37°C in the presence of 25 μc cytidine-2-C^{14} (20 mc/mmole). ‡ Actinomycin D (0.04 μg/ml) added to culture 10 minutes before pulse labeling.

The intracellular location of the RNA was monitored by autoradiography and is shown schematically in the insert of Fig. 1. As in previous experiments (*1, 9*) the labeled RNA was predominantly in the nucleoli of the control cells, whereas in the actinomycin-treated cells it was predominantly associated with chromatin. Unfortunately, quantitative data could not be obtained with these preparations because of the poor autoradiographic resolution obtainable with C^{14}. However, on similar preparations made with H^3-cytidine, the nucleoli and chromatin contained, respectively, two-thirds and one-third of the radioactivity in control cells and about one-fourth and three-fourths of the radioactivity in actinomycin-treated cells.

Varying amounts of the RNA preparations of Fig. 1 were incubated with a constant amount of DNA agar and the percentage hybridization was measured. The results are given in Table 1. For every sample of RNA tested, the percentage hybridization was much greater with the RNA from actinomycin-treated cells than it was with the RNA from control cells. To ascertain whether this difference in hybridization could be due to differences in molecular size distribution, samples of the two types of RNA preparations were degraded to the same size (approximately 10*S*) by heating at 100°C for 10 minutes and cooling rapidly (*12*). With these samples the hybridization percentages were essentially the same as those observed with undegraded material, and hence it is reasonable to conclude that the RNA's from control and actinomycin-treated cells differ with respect to specific base sequences.

The percentage hybridization obtained with a particular RNA preparation depends on the ratio of RNA to DNA in the incubation mixture (input ratio). With larger inputs of RNA there is a correspondingly lower percentage of hybridization because some of the DNA sites become saturated with complementary RNA (*13*). A quantitative comparison of two hybridization measurements must therefore be made when the input is the same. Furthermore, since the hybridization assays are based on radioactivity measurements, it is important to measure the input in terms of radioactivity. Under these circumstances variations in specific activity among the preparations are accounted for.

Measurements similar to those of Table 1 were compared over a wide range of RNA inputs. For both types of RNA the amount of hybrid increased at first linearly with input and then tended

Table 2. Competition between ribosomal RNA and pulse labeled 40*S* to 54*S* RNA from normal and actinomycin-treated cells.

Input of pulse labeled RNA* (count/min)	Unlabeled competitor RNA	Hybridization (%)	Diminution due to competitor (%)
	Normal cells		
653	None	12.6	
701	40 μg 28*S* + 16 μg 18*S*	5.0	60
1336	None	12.0	
1399	100 μg 28, 18, and 4*S*	3.7	69
1689	100 μg *E. coli* RNA†	12.1	0
	Actinomycin-treated cells		
579	None	14.1	
892	40 μg 28*S* + 16 μg 18*S*	6.2	56

* Total counts per minute recovered from agar. † Total extract.

toward saturation. Although complete saturation was not attained, there was an indication that for both types of preparation the saturating amount of DNA-RNA hybrid might be the same.

This is seen more clearly by plotting the reciprocal of the amount of hybrid against the reciprocal of the input (Fig. 2). The straight lines, calculated by the method of least squares, have the same intercept, thus indicating a common saturation level. This means that at high inputs the same number of DNA sites take part in forming hybrid and suggests that the effective hybridizing species of RNA is the component in common between the two preparations, namely the chromatin RNA. In the actinomycin-treated cells, as a result of the selective inhibition of nucleolar RNA synthesis, the chromatin-RNA accounts for a larger fraction of the radioactivity associated with newly synthesized RNA molecules. The fact that the RNA from such cells exhibits a higher percentage hybridization than does RNA from control cells indicates that with such preparations very few hybrids are formed by the nucleolar RNA.

As a consequence of its relatively low specific activity the 4*S* RNA contributes very little to the measured hybridization (*14*). Thus, the polydisperse chromatin-components appear to be the only detectable hybridizing species in unfractionated preparations of RNA. The fact that these components contain a large number of sequences complementary to DNA is consistent with the hypothesis that they constitute newly synthesized messenger RNA. In subsequently referring to them we shall use the term messenger RNA, keeping in mind however that the true criterion for messenger RNA—the role of template in the synthesis of specific proteins—has not been demonstrated in our experiments (*15*).

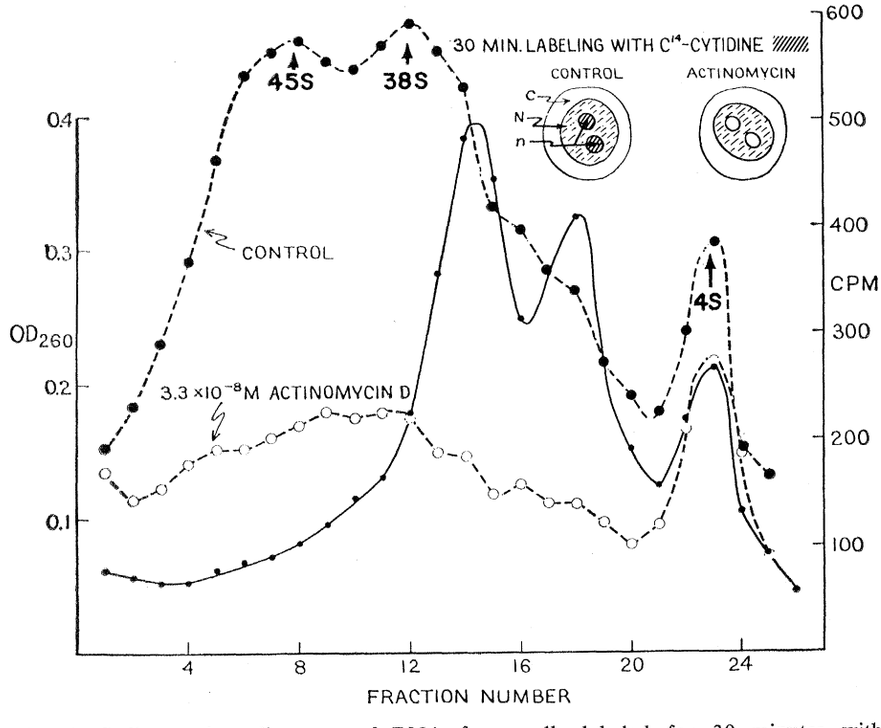

Fig. 1. Sedimentation diagrams of RNA from cells labeled for 30 minutes with cytidine-2-C^{14}. The solid curve is absorbance at 260 mμ and is essentially the same for both preparations. The peaks at fractions 14 and 18 correspond to ribosomal RNA, and the peak at 23 corresponds to 4*S* RNA. Dotted curves are counts per minute referring to control cells (closed circles) and to cells previously incubated for 10 minutes with 3.3×10^{-8} *M* actinomycin D (open circles). Sedimentation in 10 to 40 percent sucrose gradient for 3 hours at 39,000 rev/min. The inset is a schematic representation of the autoradiographs of cells from the same cultures which were used for the RNA extraction. The control cells exhibited incorporation in both the nucleoli (*n*) and the extranucleolar (chromatin) portions of the nucleus (*N*). The bulk of the incorporation in actinomycin-treated cells occurred in the chromatin region with only a sparse labeling in the nucleoli. In both cases no significant incorporation could be detected in the cytoplasm (*C*). OD, optical density; CPM, counts per minute.

The ratio of the slopes in Fig. 2 may be used to obtain a quantitative estimate of the relative fraction of messenger RNA in the control and actinomycin-treated preparations. Consider a quantity of rapidly labeled RNA, *R*, containing a fraction, *f*, of messenger RNA and a fraction, $1 - f$, of components which exhibit no detectable hybridization. If this preparation of RNA is incubated with a sample of DNA containing a number of available sites, *D*, the amount of hybrid, *H*, at equilibrium is related to *R* by:

$$\frac{1}{H} \approx \frac{1}{D}\left[1 + \frac{K}{f}\left(\frac{1}{R}\right)\right]$$

where *K* is an equilibrium constant. Thus the slope in a plot of $1/H$ against $1/R$ is inversely proportional to the fraction of messenger RNA. In Fig. 2 the ratio of the slopes is 1.8, indicating that the rapidly labeled RNA from actinomycin-treated cells is enriched by about 80 percent with respect to the messenger fraction. In two other experiments the ratio of slopes was measured to be 1.8 and 1.4.

An independent estimate of this ratio can be derived from autoradiographic and sedimentation data. In the control cells about 35 to 40 percent of the rapidly labeled RNA is chromatin RNA of which approximately one-third is 4*S* RNA. This means that roughly 23 to 27 percent of the total rapidly labeled RNA might be assumed to be messenger RNA. In the actinomycin-treated cells about 70 to 80 percent of the rapidly labeled RNA is chromatin RNA of which about two-fifths is 4*S* RNA. This implies that in such cells about 42 to 48 percent might be messenger RNA. Therefore under these assumptions one should expect an enrichment of from 1.6 to 2.1. The rather good agreement between the values estimated by the two different methods lends confidence to our interpretation of the hybridization results.

One of the most plausible explanations for the lack of hybridization of the rapidly labeled nucleolar RNA is that it contains sequences identical to those in ribosomal RNA. In this case it would be expected to form only a negligible amount of hybrid for two reasons. (i) The preparations contain a relatively large amount of unlabeled ribosomal RNA which would compete for the complementary DNA sites. (ii) The sites complementary to ribo-

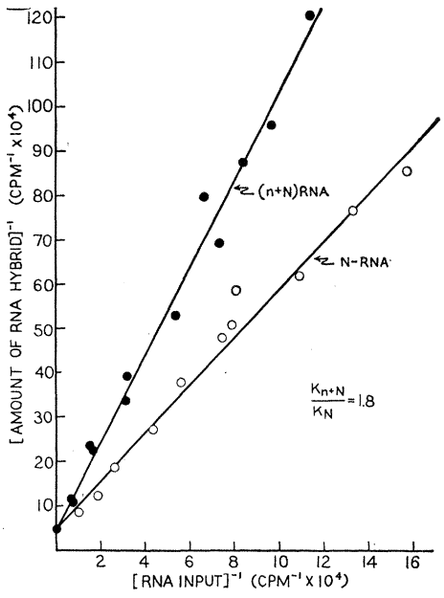

Fig. 2. Reciprocal of the amount of labeled hybrid formed plotted against the reciprocal of the input of labeled RNA. Solid and open circles correspond, respectively, to the preparations of RNA shown by solid and open circles in Fig. 1. Inputs from control cells contained from 10 to 500 μg of RNA; inputs from actinomycin-treated cells contained from 23 to 272 μg of RNA. The lines were drawn according to the method of least squares. K_{n+N}/K_N is the ratio of the slopes; n and N are defined in Fig. 1.

somal RNA are known to be few (*7, 14*) so that saturation would occur at a very low level of input. To test the validity of this proposal we prepared samples of the 38 and 45*S* components of rapidly labeled RNA which were essentially free of any contamination by ribosomal RNA. This was accomplished by first extracting the cells with phenol to remove 95 percent of the ribosomal RNA, and then extracting the rapidly labeled RNA from the interphase with phenol–sodium dodecylsulfate (*1, 2*). The heavier components of the rapidly labeled RNA were separated from the remaining 5 percent ribosomal RNA by sucrose gradient sedimentation. Samples in the 38*S* and 45*S* regions were equilibrated with double strength SSC by passage through columns (5 mm by 70 mm) of Sephadex G 25 and then incubated with the DNA agar in the presence and absence of excess amounts of unlabeled ribosomal RNA competitor.

The results, given in Table 2, show that in a range where the amount of hybrid formed is proportional to the input, excess ribosomal RNA caused a 60 to 70 percent diminution in the hybridization of pooled samples of 40 to 54*S* RNA. On the other hand, an equivalent amount of RNA from *Escherichia coli* had no effect on the hybridization, indicating that the effect of the ribosomal RNA is a true competition for specific base sequences. In the presence of excess ribosomal RNA, the hybridization of individual samples from the 38*S* and 52*S* regions was diminished by 79 and 51 percent, respectively, suggesting that the 38*S* RNA is more like ribosomal RNA than the heavier component.

Some competition was observed with RNA from actinomycin-treated cells. This result does not necessarily contradict the hypothesis that nucleolar RNA is the exclusive precursor of ribosomal RNA, since under the conditions of these experiments actinomycin does not completely inhibit nucleolar RNA synthesis. Although nucleolar RNA comprises only 20 to 30 percent of the pulse-labeled RNA in actinomycin-treated cells, it would tend to be concentrated in the 38 to 54*S* regions of the gradient. Thus, since the preparations are taken from these heavy regions of the gradient they might contain rather high proportions of nucleolar RNA which could account for the competition.

The foregoing data clearly indicate that the rapidly labeled RNA of the nucleolus contains base sequences characteristic of those in ribosomal RNA, and lend further support to the idea that the nucleolar RNA is a precursor of ribosomal RNA (*1, 2, 16*). In addition one may conclude that the transformation of precursor into ribosomal RNA occurs with preservation of base sequences. The alternative that the rapidly labeled RNA is completely degraded to mononucleotides which are then reused as the building blocks of ribosomal RNA (*17*) can be discarded.

Chipchase and Birnstiel (*18*) reported that in pea embryos ribosomal RNA hybridized as well with DNA extracted from whole nuclei as it did with DNA from isolated nucleoli, and they concluded that the cistrons for ribosomal RNA are distributed throughout the nucleus and not confined to the chromatin associated with the nucleolus. It is difficult to reconcile their findings with our results unless it is assumed that the ribosomal precursor RNA is made at various sites on the chromosomes and is then transferred to the nucleolus *as a 45S molecule* where it accumulates while being transformed into ribosomal RNA. This seems unlikely, however, since in several cell systems a high proportion of the rapidly labeled RNA is already associated with the nucleolus after precursor pulses of only a few minutes (0.001 of a cell generation) (*10, 19*).

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20. Research supported in part by NSF grants G12491 and G22140 and by NIH grant CA-06927-02. We thank B. J. McCarthy, E. T. Bolton, and B. H. Hoyer for their valuable advice concerning the agar-gel technique.
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5 June 1964