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## *DEMONSTRATION OF AN UNSTABLE RNA AND OF A PRECURSOR TO RIBOSOMAL RNA IN HELA CELLS**

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Strong evidence has accumulated in favor of the following scheme of protein synthesis in bacteria: The structural site of protein synthesis is the ribonucleoprotein particle, the ribosome.[1, 2] Amino acids in an activated form are brought by specific carrier RNA molecules (s-RNA) to the ribosomes, where organization into polypeptide chains is directed by informational or messenger RNA (m-RNA).[3–6] The bacterial DNA directs the formation of m-RNA by attracting ribonucleotides appropriate for hydrogen bonding to the bases of DNA according to the Watson-Crick model.[7] This is then followed by the enzymatic polymerization of these ribonucleotides into complementary strands of RNA.[8, 9] An important feature of the m-RNA of bacterial cells is that it is impermanent, being used in protein synthesis only 10–20 times on the average.[10] This instability of the m-RNA allows the bacterium to change quickly and efficiently its complement of enzymes by changing the type of messenger RNA that is synthesized.

Some of the elements of this scheme of protein synthesis, e.g., s-RNA and ribonucleoprotein particles are known to exist and function in animal cells. Indeed some of the earliest observations leading to the development of systems that can synthesize protein *in vitro* were made with extracts from animal tissues.[11] The nature of the informational RNA in animal cells is unclear, however. In an attempt to answer this and other questions concerning RNA from animal cells we have undertaken a study of the RNA of a strain of human cells growing in culture.

*Materials and Methods.—Cells and fractional procedures:* Suspension cultures of HeLa cells[12] growing with a generation time of 18–24 hr were used throughout. RNA was isolated by a hot phenol extraction technique;[13] DNA was prepared according to Marmur.[14] The acid soluble nucleotide pool was prepared as previously described.[15]

RNA was labeled with either uridine-2-$C^{14}$ (New England Nuclear) or $P^{32}O_4^{---}$ and was uncontaminated by DNA. DNA was labeled with thymidine-2-$C^{14}$ (New England Nuclear).

*Isotope determinations:* Radioactivity in macromolecules was determined by precipitation with 5–10 per cent cold trichloracetic acid and collection of the precipitate on membrane filters for counting. One mgm or less of solids gave little or no self-absorption of either $P^{32}$ or $C^{14}$ counts.

*Ultracentrifugal studies:* Sedimentation analysis of RNA was done by sucrose gradient sedimentation;[15] density separation was done in CsCl equilibrium density gradients.[17]

*Base analysis of nucleic acids:* Electrophoresis of ribonucleotides was performed on alkaline hydrolysates of RNA[18] in 0.05 *M* formate buffer, pH 3.5. After hydrolysis of the DNA in formic or perchloric acid[19, 20] the bases were separated by paper chromatography using isopropanol–HCl as the solvent.[21]

*Results.—Sedimentation analysis of RNA labeled for varying periods of time:* Earlier experiments had shown that when RNA from cells exposed for various times to uridine-2-$C^{14}$ was separated by sucrose gradient centrifugation two species of RNA distinct from the bulk of the cellular RNA could be detected.[13] The most rapidly sedimenting fraction (S value ~45) became labeled first; within 30–60 min after the addition of label, radioactivity appeared in the second (35S) species (see Fig. 1, control, and Fig. 2, upper left). After longer exposure times (4–24 hr), the accumulation of radioactivity in those species of RNA which comprise the

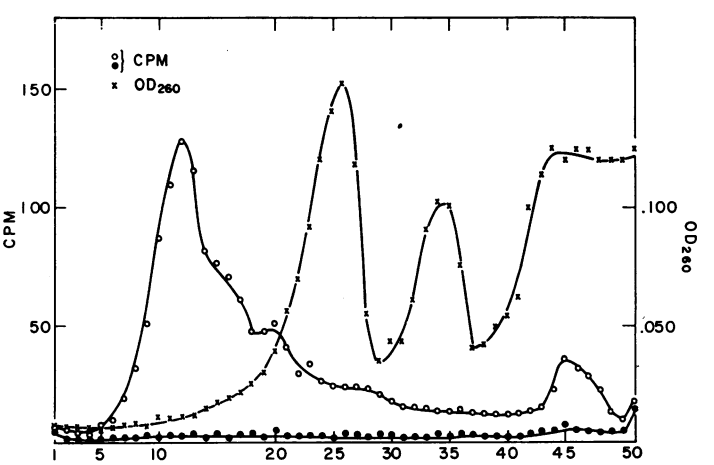


FIG. 1.—Effect of actinomycin on uridine-2-$C^{14}$ incorporation into RNA by HeLa cells. Exponentially growing HeLa cells were exposed to actinomycin D (5 $\mu$g/ml) and 10 min later uridine-2-$C^{14}$ (0.01 mM and 5 $\mu$c/$\mu$m) was added for an additional 10 min (●). Control cells not exposed to actinomycin were also labeled for 10 min (-○). RNA was extracted and examined in a sucrose gradient as previously described.[13]

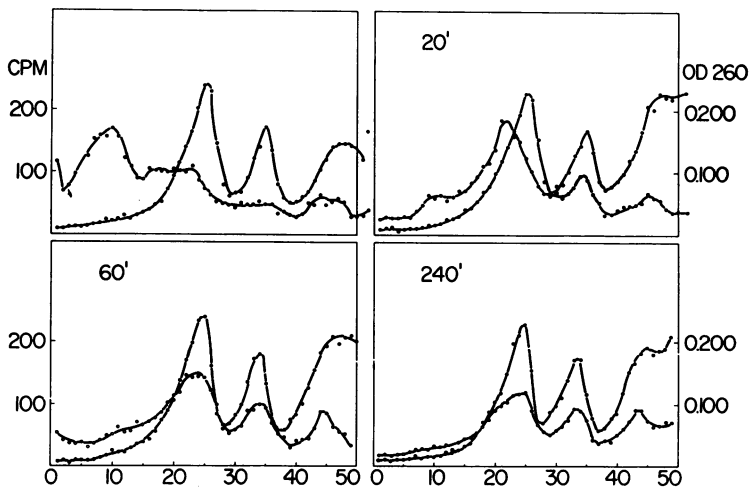


FIG. 2.—Fate of 45S RNA after actinomycin treatment. Uridine-2-$C^{14}$ (0.01 mM and 1 $\mu c$/micromole) was added to 600 ml of HeLa cells in suspension culture. After 30 min 150 ml of the culture was removed and actinomycin D (5 $\mu g$/ml) was added to the remainder. Samples of 150 ml were removed 20, 60, and 240 min after the addition of actinomycin. RNA from each sample was extracted and analyzed by sucrose gradient sedimentation (0 = CPM, ● = $OD_{260}$). See Table 3 for specific activity of the total RNA in this experiment.

TABLE 1

RECOVERY OF RNA, PROTEIN, AND DNA IN AQUEOUS PHASE AFTER HOT PHENOL EXTRACTION

| $C^{14}$ Precursor | Total CPM incorporated | Recovered after phenol extraction Total | % |
|---|---|---|---|
| Uridine | 27,800 | 20,800 | 75 |
| Thymidine | 133,000 | 135 | 0.1 |
| Valine | 235,000 | 940 | 0.4 |

Growing HeLa cells were labeled for 30 min with uridine-2-$C^{14}$ (0.5 $\mu c$/micromole), 18 hr with thymidine-$C^{14}$ (0.1 $\mu c$/micromole) or for 18 hr with valine-$C^{14}$ (0.1 $\mu c$/micromole) and extracted with hot phenol.[18] The efficiency of recovery of each label was compared by determining the acid precipitable radioactivity before and after extraction. Sucrose gradient analysis (see Fig. 1) was performed on each of the above extracts and no valine or thymidine radioactivity was present in the 35 or 45S regions where more than 70% of the uridine counts could be recovered.

bulk of the cellular RNA (28S, 16S, and 4S) obscured the relatively smaller amounts of radioactivity in the 35S and 45S material.

The extraction and sucrose gradient analysis (Table 1) of RNA from cells labeled with valine $C^{14}$ or with thymidine-2-$C^{14}$ ruled out the possibility that the large (35S and 45S) RNA was sedimenting rapidly because of attachment to or aggregation with protein or DNA. The recovery of incorporated uridine-2-$C^{14}$ in RNA was high, and the specific activity of the small amount of RNA left in the phenol phase was found not to exceed that in the aqueous phase, as has been reported to occur when different extraction procedures are used.[22]

Sedimentation analysis of the 35 and 45S materials in acetate buffer, pH 5.1, containing versene $10^{-3}$ $M$ and no $Mg^{++}$, or in tris buffer, pH 7.4, containing $10^{-4}$ $M$ $Mg^{++}$, when compared to analysis in the usual acetate buffer, pH 5.1 with $10^{-4}$ $M$ $Mg^{++}$, failed to reveal any major difference in either size or relative proportion of these materials.

*Nuclear localization of rapidly labeled RNA:* During the first hour of exposure of cells to uridine-2-$C^{14}$ at least 70–75 per cent of the incorporated activity was in the 35S or 45S fractions. Recent experiments with HeLa cells from this laboratory[23]

have indicated that almost all of the early incorporation of RNA precursors occurs in the nucleus. Hence, we conclude that the 35 and 45S RNA exists in the nucleus. This is in accord with observations on a number of different animal cell systems that the earliest incorporation of precursors into RNA is nuclear.[24, 25, 36]

*Base ratios of various RNA species:* The base ratios of the various classes of RNA (45S, 35S, 28S, 16S, and 4S) were determined by preparing from a sucrose gradient samples of each kind of RNA labeled with $P^{32}$, hydrolyzing with alkali, and analyzing the distribution of radioactivity in the resulting 2′, 3′ ribonucleotides. The validity of this method for determining base ratios has been discussed at length.[9]

The results of the base ratio analyses are given in Table 2. The outstanding

TABLE 2

BASE COMPOSITION OF HELA CELL RNA AND DNA

| Material analyzed | Sedimentation constant | % of Nucleic Acid as A | U(T) | G | C |
|---|---|---|---|---|---|
| RNA | 45 S | 19.8 | 20.1 | 29.3 | 29.8 |
| | 35 S | 19.9 | 20.7 | 28.8 | 30.4 |
| | 28 S | 20.5 | 19.3 | 28.3 | 31.9 |
| | 16 S | 23.2 | 23.2 | 25.2 | 28.0 |
| | 4 S | 22.4 | 24.5 | 24.8 | 28.3 |
| DNA, total | ... | 29.1 | 27.5 | 22.0 | 21.4 |

Various fractions of $P^{32}$-labeled HeLa cell RNA were separated by sucrose gradient sedimentation, collected, precipitated with PCA, washed with ethanol, and hydrolyzed with 0.3N KOH 16–18 hr at 37°. The resulting 2′, 3′ mononucleotides were then separated by ionophoresis at pH 3.5 in 0.05 *M* formate buffer. The radioactivity of each mononucleotide was determined as described in *Methods*. Figures given in the table represent averages of at least six determinations in each case. (A = adenylic or adenine; C = cytidylic or cytosine; G = guanylic or guanine; U = uridylic or uracil; T = thymine).

characteristic of all classes of RNA is the high (55–60 per cent) content of guanylic acid (=G) plus cytidylic acid (=C) compared to the DNA, which has only 43.5 per cent G + C. This was true of the rapidly labeled 45S and 35S material whether the time of labeling was 15, 30, or 60 min. The 28S, 16S, and 4S RNA analyzed in these experiments came from cells that had been labeled for 24 hr. Results similar to these have been reported by others for the ribosomal RNA of HeLa cells,[26] and the high G + C content has been observed consistently[27] in animal-cell RNA.

*Fate of 45 and 35S RNA:* The antibiotic actinomycin D has been shown to inhibit RNA synthesis in bacterial and mammalian cells[28] presumably by virtue of its action on DNA-dependent RNA synthesis.[29] This substance makes it possible, therefore, to investigate the fate of the early labeled RNA in HeLa cells in the absence of further RNA synthesis. Cells were labeled with uridine-2-$C^{14}$ for 30 min and then actinomycin was added to the culture at 5 $\mu$g/ml. This concentration of antibiotic effectively stops the synthesis of any further large molecular weight RNA in a few min (Fig. 1). As can be seen from Figure 2, the radioactivity from the 45S peak in the treated cultures is largely conserved and is transferred sequentially to the 35S, then to 28S and 16S fractions, which represent ribosomal RNA. The majority of the 45S and 35S RNA, therefore, appears to be precursor material to ribosomal RNA, which is itself stable.

*Unstable RNA:* The study of radioactively labeled RNA subsequent to actinomycin treatment offers the opportunity of determining the proportion of metabolically unstable RNA as well as the fate of the stable fraction. HeLa cells which have been exposed to uridine-2-$C^{14}$ for 30 min quickly lose about one-third of the acid

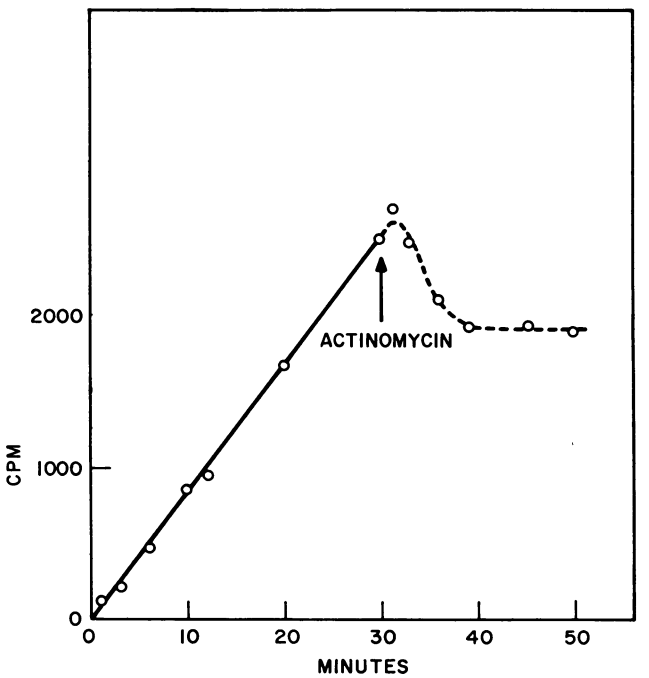


FIG. 3.—Uptake of uridine-2-$C^{14}$ into RNA of HeLa cells and the demonstration of an unstable RNA fraction.

Uridine-2-$C^{14}$ (12 $\mu$C/micromole) was added to a growing culture of HeLa cells and 1 ml samples were taken at indicated times (O), and chilled immediately to 0°. Cells were separated from the medium by centrifugation, treated with 1 ml of cold 5% trichloracetic acid, and the precipitate was collected on membrane filters for radioactivity determinations. Actinomycin D (5 $\mu$g/ml) was added to a portion of the culture at 30 min and samples were taken at intervals thereafter (- - - - - - -).

precipitable radioactivity following treatment with actinomycin (Fig. 3). The amount of this unstable fraction relative to the total RNA content of the cell can be estimated by assuming a 1.5 per cent increase of the RNA content of the cell in 30 min (generation time = 24 hr). The unstable fraction would then represent 0.5 per cent of the total RNA of the cell. Evidence has been presented by Levinthal *et al.*[10] that the messenger RNA fraction in *B. subtilis* has similar characteristics of instability.

Corroborative evidence that the loss of acid-precipitable radioactivity following actinomycin treatment of HeLa cells actually represents the loss of a fraction of RNA comes from examination of phenol extracts of labeled cells (Table 3). The total RNA from a culture that has been labeled for 30 min has a greater specific

TABLE 3

EFFECT OF ACTINOMYCIN D ON SPECIFIC ACTIVITY OF RNA EXTRACTABLE FROM HELA CELLS

| Conditions of labeling | RNA extracted: Total CPM | RNA extracted: $OD_{260}$ units | Specific activity CPM/OD unit |
|---|---|---|---|
| Uridine-2-$C^{14}$ for 30′ | 7,840 | 33.5 | 234 |
| " " " Actinomycin for 20′ | 5,780 | 39.5 | 147 |
| " " " " " 60′ | 5,020 | 33.6 | 150 |
| " " " " " 210′ | 4,660 | 33.0 | 141 |

Total RNA samples from the experiment described in Figure 3 were examined prior to sucrose gradient analysis or acid precipitable radioactivity and UV absorbancy and specific activities were calculated.

activity than the RNA from a culture which has been labeled, then treated with actinomycin. No loss of radioactivity from RNA of cells that had been labeled for 18 hr prior to treatment with actinomycin could be detected, a further indication that the unstable fraction was small relative to the total RNA of the cell.

Pertinent to the question of the sedimentation characteristics of the unstable fraction is the observation that, after a 30 min exposure to uridine-2-$C^{14}$, about 70 per cent of the total radioactivity in RNA is in 35S and 45S material (Fig. 2). Subsequent to actinomycin treatment, radioactivity accumulates in fractions of smaller size (28S, 16S, 4S). These observations suggest that most of the unstable fraction of RNA is contained in the 35 and 45S peaks.

*Stimulation of in vitro protein synthesis by HeLa cell RNA:* *In vitro* protein synthesis by *E. coli* extracts can be made dependent on the addition of RNA to the system.[30] The fraction of the bacterial RNA that has the greatest stimulatory capacity is also the RNA that is most rapidly labeled. Since this system from *E. coli* responds to many other types of RNA, it affords a means of testing the capacity of any species of RNA to serve as messenger. Various classes of HeLa-cell RNA were prepared and added to the *E. coli* system described by Nirenberg and Matthaei.[30] Table 4 shows that the 45S RNA has the greatest stimulatory capacity for the incorporation of $C^{14}$ valine into protein.

TABLE 4

STIMULATION BY HELA CELL RNA OF *in vitro* PROTEIN SYNTHESIS BY EXTRACTS OF *E. coli*

| Size of RNA | Amount ($\mu$g) | CPM incorporated above background | CPM/$\mu$g |
|---|---|---|---|
| 45 S | 36 | 48 | 1.33 |
| 35 | 97 | 57 | 0.59 |
| 28 | 136 | 55 | 0.40 |
| 16 | 125 | 54 | 0.44 |
| 10 | 78 | 25 | 0.32 |
| 4 | 163 | 13 | 0.08 |

Various classes of RNA from HeLa cells were prepared by sucrose gradient sedimentation, as described in Figure 1, and precipitated with 0.5 mg of *E. coli* S-RNA by the addition of ethanol. The precipitates were taken almost to dryness to remove the ethanol and redissolved in 0.25 ml of the *in vitro* protein synthesizing mixture described by Nirenberg and Matthaei.[30] The incorporation of $C^{14}$-valine after 30 min at 37° was determined after three extractions with 5% TCA at 95°. The background (16) represents incorporation by the extract in absence of added RNA except the s-RNA. Radioactivity determinations were made on a gas-flow counter with a background of 2 cpm and all determinations represent 1000 counts or more.

*Hybridization of HeLa RNA with DNA:* Spiegelman and co-workers[31, 32] have recently shown that both informational RNA and ribosomal RNA can attach specifically to homologous DNA by hydrogen bonding. Furthermore, it is clear from their work that in a mixture of the total bacterial DNA with the total RNA (informational RNA as well as ribosomal RNA) there is a much greater chance for hybridization of the informational RNA. This implies that in the bacterial cell the portion of DNA which functions in the formation of informational RNA is much larger than that concerned with the production of ribosomal RNA.

If in HeLa cells the number of loci which produce ribosomal RNA is small relative to the total number of gene loci, and if the DNA that is functioning in these cells (other than that responsible for ribosomal RNA) has the average composition of the total DNA, then hybridization of pulse-labeled RNA from these cells should reveal any DNA-like RNA.

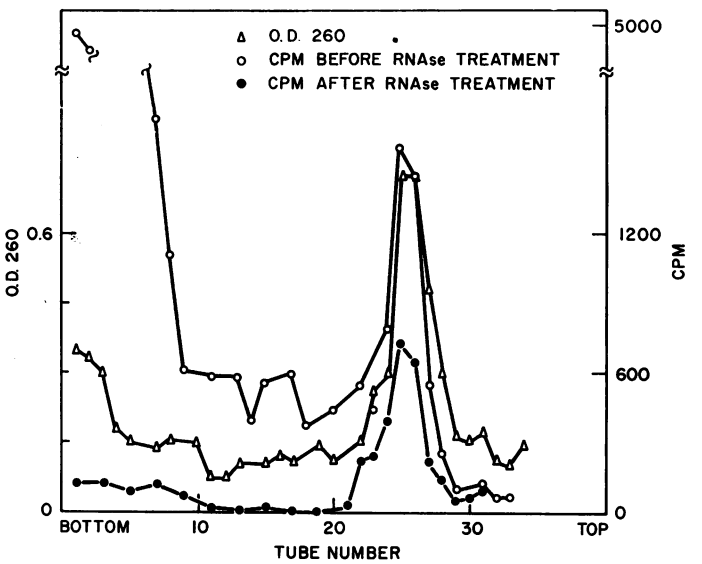


FIG. 4.—Hybridization of HeLa cell RNA with DNA. RNA labeled with $P^{32}$ from the 35S region of a sucrose gradient was collected by alcohol precipitation and dissolved in saline citrate buffer (0.3 *M* NaCl and 0.03 *M* Na citrate, pH 7.8). This was mixed with 0.8 mg of HeLa cell DNA which had been heated to 100°C for 15 min and cooled to 0° in 30 sec. The DNA-RNA mixture was brought to 60° and slowly cooled over 36 hr to 28°. After dilution with saline-citrate buffer, solid CsCl was added to give a density of 1.765 in a final volume of 4.5 ml. The material was then analyzed by density gradient sedimentation in a model L Spinco ultracentrifuge (SW 39 rotor, 35,000 rpm for 60 hr). The ribonuclease treatment was performed by diluting samples from the gradient 30-fold in buffer containing $5 \times 10^{-3}M$ tris, $10^{-4}M$ Mg $Cl_2$, and digesting with 5 μg ribonuclease for 30 min at 37°.

TABLE 5

COMPOSITION OF HYBRIDIZED FRACTION OF RNA

| | G + C Content | |
|---|---|---|
| | *Total before hybridization* | *Hybridized fraction* |
| 35 and 45S RNA | 59.2 | 50.5 |
| Total RNA | 59.5 | 46.0 |
| DNA | 43.6 | ... |

RNA from the experiments described in Figure 4 analyzed for base composition as in Table 2. Only total G + C content is presented in order to facilitate comparisons.

The results of experiments of this type are given in Figure 4 and Table 5. It is clear that RNA labeled for 60 min with $P^{32}$ can hybridize with HeLa cell DNA forming a complex that resists digestion by ribonuclease, the criterion of a true hybrid according to Hall and Spiegelman.[31] The base ratio of the hybridized RNA is significantly lower in G + C than the total RNA or the 35 and 45S RNA, an indication that there is in the 35 and 45S material an RNA fraction resembling DNA in base composition mixed with the ribosomal type RNA. Further work will be necessary to determine whether only molecules of RNA with low G + C content (DNA-like) are hybridized or whether the hybridized material is still a mixture of high G + C (ribosomelike) and low G + C RNA.

*Discussion.*—The following conclusions can be drawn from the experiments reported in this paper: (1) Ribosomal RNA in the HeLa cell is formed as part of very large RNA molecules which are then converted into smaller pieces. (2) There

is in these cells a fraction of RNA that is rapidly synthesized and degraded, but the majority of the RNA is metabolically stable. (3) Of the total RNA a small fraction, which preferentially hybridizes with DNA, has a base ratio nearer to that of DNA than the majority of the RNA. This fraction possibly corresponds to the DNA-like RNA described by others in animal cells.[33, 34]

The first question raised by these experiments is the molecular nature and significance of the very large RNA molecules which are chiefly, if not entirely, precursors of ribosomal RNA. Several lines of evidence indicate that these molecules are not artifacts. Their size is extremely reproducible; the 45S is always formed first, followed by the 35S, then 16S and 28S; the absence of divalent cations seems not to affect their size; these fractions are uncontaminated with protein or DNA; the procedure used to isolate the RNA—heating with phenol to 60° three times for 3 minutes and cooling to −5° in 30 seconds—should not allow the type of complementary pairing of RNA which has been reported by Geiduschek *et al.*[35] Moreover, RNA of similar size has now been found in animal cells of several different types.[36]

What is the meaning of 45S RNA? One possibility is that several ribosomal RNA cistrons lie together and the 45S material is a complex of several ribosomal RNA molecules. This arrangement of ribosomal cistrons in bacteria has been suggested by Yankofsky and Spiegelman[32] to explain some properties of the bacteria DNA-ribosomal RNA hybrids. Implicit in this scheme is a mechanism for accurately dividing the large molecule into smaller ones of specific sizes.

The second problem to which this work is pertinent is the question of whether there is in animal cells any protein synthesis governed by a species of RNA analogous to messenger RNA in bacteria. The observations that the RNA from HeLa cells can stimulate *in vitro* protein synthesis by *E. coli* extracts, that an unstable fraction of RNA exists, and that a small fraction of the total RNA has DNA-like base ratios suggest that messenger RNA does indeed exist in animal cells. Further work aimed at isolating the unstable fraction in order to determine its composition, intracellular localization, and function will be necessary before a definite conclusion can be drawn concerning not only the existence but also the quantitative role of an unstable messenger RNA in protein synthesis by animal cells.

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## *TRANSFER OF CHLOROPHYLL EXCITATION ENERGY IN GREEN PHOTOSYNTHETIC BACTERIA**

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Beginning with Katz and Wassink,[1] several investigators[2–4] have obtained from suspensions and extracts of green photosynthetic bacteria, absorption spectra which suggest the presence of a minor chlorophyll component in addition to the major characteristic pigment, chlorobium chlorophyll (bacterioviridin). The fluorescence emission spectra of whole cells recently observed by Krasnovskii *et al.*[5] are even more suggestive in this respect than the absorption spectra. Isolation of the partially purified minor chlorophyll was accomplished by Olson and Romano.[6] This pigment, which has a striking spectroscopic resemblance to bacteriochlorophyll, was named "chlorophyll-770" because of the position of its red absorption peak in ether. *In vivo* the absorption band at about 810 mμ due to chlorophyll-770 is only 5 to 10 per cent of the main red band due to chlorobium chlorophyll. To test the suggestion[6] that the predominant chlorobium chlorophyll acts as an accessory pigment which transfers excitation energy to the minor component, chlorophyll-770, we have investigated the fluorescence of the protein-chlorophyll-770 complex *in*