necrosis after 166–227 days. There was a concomitant, yet statistically not significant, increase in the uptake of RNA precursors, also antedating the manifestation of delayed necrosis. The findings are considered to be the possible result of chromosomal radiogenic injury or of a genetic injury to the RNA synthesizing cellular systems.

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*A RIBONUCLEIC ACID FRACTION FROM RAT LIVER WITH TEMPLATE ACTIVITY**

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The messenger RNA theory, developed on the basis of observations with microorganisms,[1, 2] is concerned with a species of RNA with the following properties: a rapid rate of turnover, a nucleotide composition similar to that of the DNA of the cell, and the ability to stimulate the incorporation of amino acids into protein by ribosomes. Because of its high turnover, this RNA can be selectively labeled in microorganisms by subjecting the cells to a short pulse of radioactive precursors of RNA.[3] Most of the studies of this material have been done with whole cellular RNA from pulse-labeled cells. In mammalian cells, however, no pulse-labeled RNA with a DNA-like composition has been detected.[4, 5] The study of mammalian RNA species analogous to the messenger RNA of bacteria will therefore require their physical separation from the bulk of the cellular RNA. Recent studies have indicated that RNA with a DNA-like composition remains in the gel interphase when nuclear fractions of mammalian cells are subjected to the phenol procedure for RNA extraction.[6, 7] This RNA can be recovered by re-extracting the interphase at elevated temperature.[7] Extraction of the interphase with slightly alkaline buffers (pH 8.5 and 9.0) also yields a RNA species with a nucleotide composi-

tion somewhat similar to that of the DNA.[8] The present study is based on the finding that the material isolated from rat liver by the latter method is highly active with respect to stimulation of amino acid incorporation into protein by preincubated *Escherichia coli* ribosomes.[9] The nucleotide composition of this RNA species, although approaching that of rat DNA, differs significantly from it, particularly with respect to adenylic acid content.

The possible physiological function of this RNA fraction is suggested by its ability to stimulate the activity of *E. coli* ribosomes, a property that could be expected of a template for protein synthesis. Evidence for such a template activity has been presented in the case of viral RNA added to the *E. coli* ribosomal system.[10] The identification of the rat liver material as "template RNA," however, can only be tentative in the absence of experimental evidence that it serves as template for the formation of specific proteins. The designation "T-RNA," will be used for this RNA fraction.

Materials.—Pyruvate kinase was obtained from Biochemica-Boehringer, DNase (1 × crystallized) from Worthington Biochemical Co., and uniformly labeled L-leucine-C^{14} and L-phenylalanine-C^{14} from Schwarz BioResearch, Inc. The tricyclohexylammonium salt of phosphoenolpyruvate, purchased from Calbiochem, was converted to the sodium salt before use. Poly U was a product of Miles Laboratories, Inc.

Methods.—Cell fractionation: Fresh rat liver was disrupted rapidly in 3 volumes of 10% sucrose and 0.01 *M* Tris-HCl buffer pH 7.6 in an all-glass tissue grinder, and the suspension centrifuged at 500 × *g* for 10 min. All operations were carried out at 4°. The pellet was resuspended in 10% sucrose, 0.01 *M* Tris pH 7.6, and 0.001 *M* $MgCl_2$, and collected by centrifuging at 500 × *g*. This crude nuclear fraction served for the phenol fractionation. The 500 × *g* supernatant was centrifuged at 100,000 × *g* for 2 hr. The resulting supernatant served for the preparation of the "supernatant" RNA. The 100,000 × *g* pellet was suspended in 0.5% DOC, 0.01 *M* Tris pH 7.6, and 0.002 *M* $MgCl_2$, and the suspension centrifuged at 12,000 × *g* for 30 min. The ribosomes were sedimented from the 12,000 × *g* supernatant at 100,000 × *g* for 2 hr. The resulting supernatant served for the preparation of the "DOC supernatant" RNA.

RNA fractions: The nuclear RNA fractions were obtained by a procedure described previously.[8] In this procedure ribosomal RNA is first removed by a phenol extraction at pH 7.6, and T-RNA fractions are obtained at pH 8.5 and 9.0, together with some DNA. The supernatant fractions were adjusted with Tris buffers of pH 7.6 or 9.0 to 0.1 *M*, and the ribosomal pellet was suspended in 0.1 *M* Tris pH 9.0. These were mixed with equal volumes of chilled phenol (redistilled), stirred gently at room temperature for 1 hr, and the aqueous phases recovered by centrifugation. RNA was precipitated from the aqueous extracts by addition of 0.1 volume of 10% NaCl and 2.5 volumes of ethanol. The precipitates were dissolved in small volumes of ice-cold water and adjusted with NaCl to a concentration of 10%. Storage at 4° for 1–2 days brought about the precipitation of high molecular weight RNA. This was recovered by centrifugation. When needed, the RNA soluble in NaCl was obtained from the supernatant by precipitation with 2 volumes of ethanol. For the incorporation experiments, the pellets were freed of contaminating NaCl by suspension in ice-cold 66% aqueous ethanol and recentrifugation. The well-drained pellets were dissolved in water. All the RNA preparations used in this study were stored in the deep-freeze.

E. coli sRNA was prepared from frozen cells suspended in cold 0.1 *M* Tris pH 7.6 by a procedure described previously.[11] The sRNA was purified by dialysis against water, then lyophilized, and redissolved in water for storage.

E. coli S-30 preincubated extract was obtained by the procedure of Matthaei and Nirenberg[9] with two modifications. The cells were disrupted in the French Press at 10,000 lbs/in^2, and sRNA was omitted from the preincubation mixture. The conditions described by Matthaei and Nirenberg[9] for the amino acid incorporations were used, but both UTP and CTP were omitted. The method of Mans and Novelli[12] was used to determine the amounts of radioactive amino acid incorporated into protein.

Analytical methods: The nucleotide composition of the RNA samples was determined as described previously.[13] Total RNA was determined either from the nucleotide composition data, or by absorbancy measurement at 260mμ of samples treated with 0.5 *N* perchloric acid at 70° for 20 min.[14] Protein was determined as described previously.[14]

Results.—Nucleotide composition of T-RNA: Table 1 summarizes the results of two fractionation experiments with a crude rat liver nuclear preparation. The pH 7.6 extracts, which comprise the bulk of the RNA, have the same nucleotide composition as the RNA obtained from the purified ribosomes. The nuclear fraction appears to be contaminated with a considerable amount of ribosomal material, as judged by the large proportion of the cellular RNA usually recovered in this fraction (about 30%). Extraction at pH 8.0, which follows several extractions at pH 7.6,[8] yields RNA with a nucleotide composition somewhat shifted from the ribosomal values. The material obtained at pH 8.5 and 9.0 shows a sharply different nucleotide composition, with the value for each nucleotide shifted toward the corresponding DNA value. The total amounts of T-RNA (fractions 8.5 and 9.0) obtained were quite variable (see Table 1). The highest yield obtained (Prep. II) amounted to about 1 per cent of the total liver RNA.

In Figure 1, the A, C, and U contents of the various RNA fractions are plotted against the corresponding G content. The straight line relationships obtained suggest that the fractionation procedure resolves a mixture of two components, ribosomal RNA and a RNA species with a nucleotide composition somewhat similar to that of DNA. Although no further change in nucleotide composition is observed after repeated extractions at pH 8.5 and 9.0 (Table 1), it is quite probable that the T-RNA fractions still contain a substantial amount of ribosomal RNA. Only the

TABLE 1

PHENOL FRACTIONATION OF RAT LIVER NUCLEAR RNA

	Preparation I				Preparation II			
	pH 7.6	pH 8.0	pH 8.5	pH 9.0	pH 8.5_1	pH 8.5_2	pH 9.0†	DNA*
A	18.0	19.8	24.5	24.9	25.8	25.5	25.4	28.6
G	32.8	31.8	28.8	29.0	27.9	26.3	26.4	21.4
C	31.2	30.2	26.8	25.6	25.1	26.3	26.7	21.5
U	18.0	18.1	19.8	20.5	21.1	21.6	21.6	28.4
RNA, yield		1.5	0.5	1.6	5.9	3.5	3.2	

* See ref. 15; C value represents sum of cytidylic and 5-methylcytidylic acid contents; U value stands for thymidylic acid content.

† Material could not be precipitated with NaCl (see text).

Starting material for Preparation I, 45 gm of liver, and for Preparation II, 60 gm. Headings pH 8.5_1 and 8.5_2 refer to first and second extraction at pH 8.5. Nucleotide composition data are expressed as moles per 100 moles of total nucleotides; RNA yields are expressed in μmoles nucleotide.

A content of these fractions is relatively close to that of DNA. The U content, on the other hand, shows only a modest shift away from the ribosomal value. These unequal shifts indicate that the nucleotide composition of the T-RNA component must deviate appreciably from that of DNA. It probably possesses a higher A content together with a lower U content.

The data of Table 1 for all but one of the fractions (pH 9.0, Prep. II) apply to high molecular weight RNA precipitated with 10 per cent NaCl in the course of purification. Since DNA is not precipitated under these conditions, this step results in the removal of DNA from the T-RNA fractions. In some instances, particularly when much DNA was present, very little or no precipitate could be obtained with NaCl. The material recovered from the supernatant, however,

contained RNA with the expected composition (see fraction 9.0 of Prep. II). Even where the salt precipitation was successful, about half the RNA remained soluble in the NaCl solution. In all cases this soluble material had the same nucleotide composition as the salt-precipitated RNA. Although the soluble material could represent degraded T-RNA, it is also possible that the DNA present interferes with the salt precipitation. Unless noted otherwise, only the salt-precipitated RNA was used in the subsequent experiments.

The sharp differences in nucleotide composition between the T-RNA fractions and ribosomal RNA provided a convenient means for identifying these fractions without nucleotide analysis. As can be seen in Table 2, the UV absorption spectra in acid solution of hydrolyzed samples of the two types of RNA are quite different. The ratios of absorbancies listed in Table 2 were used to check the composition of the pH 8.5 fraction from Preparation III, which was used in some of the incorporation experiments. The absorbancy ratios of this material were 0.39 and 0.68. This simple test can be used only when no contaminating DNA is present.

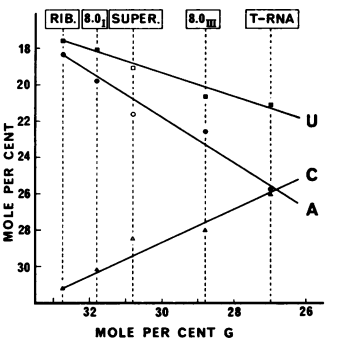

FIG. 1.—Variations in nucleotide composition of rat liver RNA fractions. The values for each fraction are connected by dashed vertical lines. Ribosomal values (RIB.) are the mean for two preparations of pH 7.6 nuclear RNA; T-RNA values are mean for all the pH 8.5 and 9.0 fractions studied. Two different sets of values for the pH 8.0 fractions are plotted; one was obtained from Prep. I, and the other from III. The supernatant RNA (SUPER) values described in Table 2 are used.

Cytoplasmic RNA fractions: In order to detect the possible presence of T-RNA in the cytoplasm, two high molecular weight RNA fractions were examined with respect to nucleotide composition (Table 3). The RNA of the 100,000 $\times$ *g* supernatant consistently showed small shifts compatible with the presence of some T-RNA together with ribosomal RNA. The nucleotide composition of this material fits into the linear relationships of Figure 1. As will be seen below, this fraction also shows template activity. The same material could be obtained by

TABLE 2

SPECTRAL RATIOS OF RIBOSOMAL AND T-RNA IN ACID SOLUTION

	A 290 mμ / A 260 mμ	A 280 mμ / A 260 mμ
Ribosomal RNA	0.43	0.73
T-RNA	0.39	0.66

Alkaline hydrolysates of RNA diluted with 0.5 *N* $HClO_4$. Template RNA of Preparation III (pH 8.5 fractions) was used.

TABLE 3

NUCLEOTIDE COMPOSITION OF CYTOPLASMIC HIGH MOLECULAR WEIGHT RNA FRACTIONS

	100,000 × *g* supernatant		Microsomal DOC supernatant
A	21.6	(0.7)	20.8
G	30.8	(0.2)	32.5
C	28.5	(0.7)	29.5
U	19.1	(0.8)	17.2
RNA, yield	5.1	(4.2)	25

Values for the 100,000 × *g* supernatant represent the mean values for 3 preparations; numbers in parentheses are standard deviations. Only 1 preparation of the DOC supernatant was analyzed. RNA yield expressed as μmoles nucleotide per 60 gm liver.

phenol extraction at pH 7.6 as well as at pH 9.0. Furthermore, re-extraction at pH 9.0 of the nonaqueous residue left after extraction at pH 7.6 did not yield any additional RNA.

The microsomal DOC supernatant RNA showed a slightly elevated A content, but no other shifts toward a DNA-like composition were evident. This material showed no template activity.

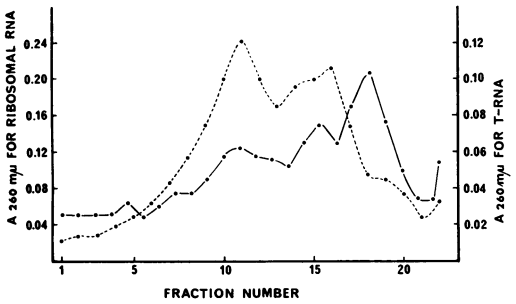

FIG. 2.—Zone centrifugation patterns for T-RNA (solid line) and ribosomal RNA (dashed line). Material was placed on top of a 5–20% sucrose gradient in 0.01 *M* Tris pH 7.6 and 0.001 *M* $MgCl_2$, and centrifuged at 35,000 rpm for 5 hr in the SW39 Spinco rotor. 18 drop fractions were diluted with H_2O to 3 ml before UV measurements. pH 7.6 and 8.5 fractions of Prep. III were used in this experiment.

Sedimentation characteristics of T-RNA: Figure 2 shows the patterns obtained by zone centrifugation of T-RNA and ribosomal RNA preparations. The T-RNA pattern shows two small peaks, which correspond to the peaks of ribosomal RNA, and a major component lighter than ribosomal RNA. If the sedimentation values of the ribosomal components are considered to be 18 and 26S, then, by extrapolation, a value of approximately 10S can be estimated for the light T-RNA component. Of the three components of the T-RNA preparation, the two heavy ones, which correspond to the two components of the ribosomal RNA preparation, may represent contaminating ribosomal material. The 10S component, which is not evident in the ribosomal RNA preparation, may contain the material responsible for the template activity.

TABLE 4

CHARACTERISTICS OF STIMULATION OF LEUCINE INCORPORATION BY T-RNA

	Leucine incorporated (CPM/ml) Prep. II	Prep. III
Complete system	4,850	1,340
Minus T-RNA	60	180
Plus DNase	4,280	
Plus chloramphenicol	180	
Plus puromycin	40	
Plus sRNA		1,690
Minus amino acid mixture		690

Complete system contained 260 μg/ml of pH 8.5 fraction of Preparation II, and 140 μg/ml of pH 8.5 fraction of Preparation III; for other details see Fig. 3. 80 μg/ml each of DNase, chloramphenicol, and puromycin, and 1 mg/ml of sRNA, were used.

Template activity of RNA fractions: The incorporation of leucine into protein by *E. coli* ribosomes is strongly stimulated by the T-RNA fractions (Fig. 3). The extent of stimulation increases linearly with increasing amounts of T-RNA, except at very low concentrations (20 μg/ml) where no effect is observed. The concentration curves for two different RNA fractions with similar nucleotide compositions were very close to each other. The slight activity of the ribosomal RNA also appears to rise linearly. No inhibition is evident up to a concentration of 1 mg/ml. The concentration curve for the stimulation of phenylalanine incorporation by poly U is quite different from those obtained with the liver RNA fractions.

In contrast to the behavior of the latter (see below), the poly U-stimulated incorporation was highly dependent on added sRNA. At the concentration of 160 μg/ml, the activity of poly U for phenylalanine is 10 times greater than that of liver T-RNA for leucine. Since all 20 amino acids must be incorporated with T-RNA, however, the specific activities of both poly U and T-RNA may be similar.

The incorporation stimulated by liver T-RNA showed the high sensitivity to chloramphenicol and puromycin characteristic for the *E. coli* ribosomal systems. Addition of DNase produced a slight inhibition, perhaps due to the presence in the DNase preparation of some contaminating ribonuclease. The sRNA present in the *E. coli* preparation appeared nearly sufficient for maximal incorporation of leucine, since additional sRNA produced only a slight increase. Omission of the complete amino acid mixture reduced the extent of incorporation by about 50 per cent. Kinetic experiments indicated that the incorporation was nearly completed after 15 min.

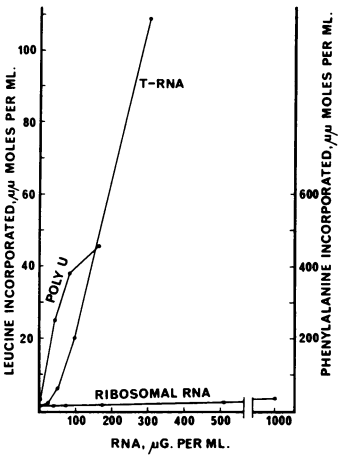

FIG. 3.—Template activity of rat liver RNA fractions. Incubation mixtures contained 2.2 mg/ml of S-30 protein and 0.75 mg/ml of S-30 RNA. In experiments with poly U, 1 mg/ml of sRNA was included. 0.04 μmole/ml of leucine or phenylalanine (40 μcuries/μmole) were used. The T-RNA was from Prep. II (pH 8.5_1); the nuclear pH 7.6 fraction was used as ribosomal RNA. Leucine was used for the experiments with the liver RNA fractions, and phenylalanine for the experiments with poly U.

Since the stimulating effect of the RNA fractions was linear over a wide range of concentrations, a comparison of the activity of different types of RNA was possible (Table 5). The ribosomal RNA preparations showed about 1 per cent of the activity of the T-RNA fractions. The supernatant RNA, whose nucleotide composition indicated the possible presence of some T-RNA, showed about 3 times the activity of ribosomal RNA; the pH 8.0 fraction with a nucleotide composition clearly intermediate between that of T-RNA and ribosomal RNA, showed a substantial activity. The RNA of the pH 9.0 extract, which could not be precipitated with NaCl, still showed considerable activity. Since this fraction was heavily contaminated with DNA, it was necessary to test the possibility that its activity is due to the DNA. Only a small inhibition was observed when DNase was added to

TABLE 5

TEMPLATE ACTIVITY OF LIVER RNA FRACTIONS

RNA fraction	RNA concentration (μg/ml) Prep. II	Prep. III	Specific activity* Prep. II	Prep. III
Nuclear pH 7.6	510	960	3	4
" pH 8.0		210		38
" pH 8.5_1	300	140	360	208
" pH 8.5_2	310		300	
" pH 9.0	260		132	
" pH 9.0 + DNase†	260		111	
Ribosomal		910		4
DOC soluble		950		2
Supernatant	500	890	10	12

* Specific activity refers to amount (in $\mu\mu$moles) of stimulation of leucine incorporation per mg of added RNA.

† 80 μg/ml of DNase.

the incubation mixture. A T-RNA fraction, which had been inadvertently degraded to an average value of 5S, showed very little template activity. This indicates that the RNA fractions not precipitable by NaCl must still be of relatively high molecular size.

Competition experiments between ribosomal RNA and T-RNA: In order to determine whether ribosomal RNA exerts an inhibitory effect on the activity of T-RNA, mixtures in varying proportions of the two RNA species were tested with the *E. coli* ribosomal system. Even in the presence of a 25-fold excess of ribosomal RNA, no inhibition was evident. Since the T-RNA preparations were probably about 50 per cent pure, the amount of T-RNA in this mixture may have been around 2%. There appeared to be an enhancement of the activity of the T-RNA, possibly because of a protection of the latter from ribonuclease action. In this experiment, the amount of ribosomal RNA added was equal to the total RNA of the S-30 preparation. These results indicate that ribosomal RNA does not compete with T-RNA for the *E. coli* ribosomal sites. When a 50-fold excess of ribosomal RNA was used, an inhibition of T-RNA was evident. Because of the small amount of T-RNA used, however, the levels of incorporation were rather low and the result of this last experiment may not be very conclusive.

TABLE 6

EFFECT OF RIBOSOMAL RNA ON STIMULATION OF INCORPORATION BY T-RNA

Template RNA (μg/ml)	Ribosomal RNA (μg/ml)	Leucine incorporated (CPM/ml)	Specific activity* of T-RNA
—	—	110	
—	890	310	
—	1,900	440	
140	—	1,340	220
140	890	1,630	235
81	—	820	220
81	1,900	1,400	295
41	—	330	135
41	1,900	540	60

* Specific activity of mixed incubations computed as follows: stimulation due to ribosomal RNA alone deducted from total stimulation, and value obtained divided by concentration of T-RNA; values expressed as μμmole leucine incorporated per mg of added RNA.

Nuclear pH 7.6 fraction (Prep. III) used as ribosomal RNA, and nuclear pH 8.5 fraction from same preparation used as T-RNA. Amount of S-30 used per ml incubation mixture: 6.6 mg protein, 1.9 mg RNA.

Discussion.—The present results demonstrate the presence in rat liver of a species of RNA with some of the characteristics ascribed to the bacterial messenger RNA. Its composition is somewhat similar to that of DNA and it stimulates strongly the incorporation of leucine into protein by the preincubated *E. coli* ribosomal system. This material appears to be localized primarily in the nucleus. It is most probably this cellular localization which forms the basis for the fractionation procedure used here. Of the various cytoplasmic RNA fractions, only the high molecular weight RNA of the 100,000 x *g* supernatant may contain any significant amount of T-RNA, as judged by both nucleotide composition and template activity. It cannot be stated at present whether this material is normally present in the cytoplasm or whether it is leached out of the nuclei during the cell fractionation.

A recent report describes a RNA species complementary to mouse liver DNA.[16] The nucleotide composition of this material, based on P^{32}-isotopic dilution analysis,

appears to be similar to that of the RNA described in the present study. This mouse liver RNA was also shown to be present in the cytoplasm as well as the nucleus.

In our studies, the RNA extracted from the purified liver ribosomes shows a very low template activity. Ribosomes isolated in this manner are usually active in protein synthesis and, furthermore, show little or no stimulation by polyribonucleotides.[17] The data presented here suggest that the amount of T-RNA possibly present in the ribosomal extract cannot be higher than 1–2 per cent. Such an estimate is made possible by the apparent lack of inhibition of the activity of T-RNA in the presence of a large excess of ribosomal RNA.

The inability of ribosomal RNA to stimulate the activity of the *E. coli* ribosomal preparation deserves comment. The results of the competition experiments indicate that ribosomal RNA is unable to bind to the sites on the ribosomes at which T-RNA becomes attached. There must be, therefore, a basic structural difference between T-RNA and ribosomal RNA. One possibility is the difference in molecular size (10S versus 18 and 26S). It is also possible that a specific sequence of nucleotides governs the affinity of RNA molecules toward the ribosomes. The high G content of ribosomal RNA molecules toward the ribosomes. The high G content of ribosomal RNA suggests the presence of areas rich in G which could play a role in preventing the binding of the RNA to ribosomes. On the other hand, the activity of the template RNA could be determined by its apparent high A content. The possibility of the isolation of purified T-RNA in quantities sufficiently large for structural studies, as indicated in this report, may lead to some understanding of the basis for the biochemical activity of this material.

Summary.—A RNA species with a nucleotide composition somewhat similar to that of DNA has been isolated from a crude rat liver nuclear preparation. This material is 100 times more effective than ribosomal RNA in stimulating the incorporation of leucine into protein by a cell-free preparation of *E. coli*. Its activity is not inhibited by the presence of a large excess of ribosomal RNA. RNA preparations from the cytoplasm show very little template activity.

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The following abbreviations are used: DNase, deoxyribonuclease; poly U, polyuridylic acid; Tris, tris (hydroxymethyl) aminomethane; DOC, sodium deoxycholate; sRNA, amino acid transfer RNA; A, adenylic acid; G, guanylic acid; C, cytidylic acid; U, uridylic acid.

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*FAST KINETICS OF UNPAIRED ELECTRONS IN PHOTOSYNTHETIC SYSTEMS**

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Since the initial observation of an electron spin resonance (ESR) signal in chloroplasts, which increases in intensity on illumination,[1] this technique has added significantly to the data on the photosynthetic process.

The ESR signals under discussion are characterized by g values in the range of 2.002–2.007 and by widths, measured between points of maximum slope, in the range of 1–20 gauss. Such signals may be produced by organic free radicals or by unpaired electrons trapped in an extensive lattice, as in a semiconductor. They are not of the type produced by paramagnetic ions of the transition elements, or by molecules in triplet states.[2] For purposes of the following discussion, we attribute the observed ESR signals to "unpaired electrons," giving this term the meaning of implying either organic free radicals (relatively small molecules containing an unpaired electron) or electrons in semiconducting lattice situations, but excluding transition metal ions and triplet molecules.

ESR signals with g values of 2.002–2.005 have been observed in a number of photosynthetic systems.[3–10] In the anaerobic photosynthetic bacterium *R. rubrum*, no signal is observed in the dark, but an intense signal at $g = 2.002$ is observed on illumination. In green plants which produce O_2 photosynthetically, two signals have been observed. One of these (signal II, ref. 5) is observed in the dark; it is centered at $g = 2.005$ and exhibits distinctive hyperfine lines. A second signal (signal I, ref. 5) is absent in the dark and is generated on illumination; this signal has a g value of 2.002 and does not exhibit hyperfine structure. ESR studies of photosynthetic systems have not thus far yielded unequivocal evidence of unpaired electrons associated with semiconductors or with triplet excited states.

The main question under examination in the present investigation is whether the observed unpaired electrons are associated with a component representing the primary light-excited state, or with some later stage in the photosynthetic process. For this purpose, we have studied fast kinetic changes in the intensity of the ESR signal at $g = 2.002$ exhibited by intact cells of *R. rubrum* on illumination. Until