Amino Acid Incorporation and Products of Protein Synthesis in Isolated Chloroplasts of *Euglena gracilis*†

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ABSTRACT: An improved isolation procedure has resulted in good yields of purified chloroplasts from the alga, *Euglena gracilis*, with high activity in protein synthesis *in vitro*. Amino acid incorporation is supported by exogenously added ATP or by photosynthetic phosphorylation and is sensitive to antibiotics which inhibit bacterial protein synthesis, insensitive to cycloheximide, and only slightly sensitive to actinomycin D and rifampicin. Since nucleoside triphosphates required for RNA synthesis do not stimulate protein synthesis, concomitant RNA synthesis may not be necessary for maximal levels of protein synthesis in these chloroplasts. Products of this protein synthesis, analyzed by column chromatography and acrylamide gel electrophoresis, include both soluble and "structural" proteins of a wide range of molecular weights. Among these is a soluble fraction which comigrates with ribulose diphosphate carboxylase activity on agarose column chromatography. Ferredoxin and cytochrome *c* 552 are not detectably labeled. Comparison with chloroplast proteins synthesized *in vivo* when cytoplasmic ribosomes are inactivated by treatment with cycloheximide indicates that the protein synthesis observed *in vitro* is faithful to the patterns of synthesis on chloroplast ribosomes *in vivo*.

Chloroplasts possess their own DNA and a self-contained protein-synthesizing machinery distinct from that of the cytoplasm. Although synthesis of nucleic acids and proteins can occur in isolated chloroplasts (see Kirk, 1970, for references), most reports of the products of chloroplast protein synthesis have been derived indirectly from studies *in vivo* or from genetic studies. Attempts at direct analysis of products of chloroplast protein synthesis *in vitro* have yielded interesting but contradictory results (Chen and Wildman, 1970; Goffeau, 1969; Margulies, 1970; Margulies and Parenti, 1968; Ranalletti *et al.*, 1969; Spencer *et al.*, 1971).

The development of a large-scale method of preparing physiologically active chloroplasts from *Euglena gracilis* (Preston *et al.*, 1972) and the establishment of conditions under which these chloroplasts actively incorporate radioactive amino acids into protein has made it possible to carry out fractionation of newly synthesized proteins. We describe here the principal characteristics of this system and present data on direct biochemical analysis of its products, among which are both soluble and lamellar proteins of a wide range of molecular weights.

Methods

Cells were cultured and chloroplasts isolated as described by Preston *et al.* (1972), with the following modifications. Cells were harvested when the pH of the culture reached 7.0–7.2, corresponding to early stationary phase in the growth curve. The concentration of $MgCl_2$ in the STM buffer[1] was raised from 0.004 to 0.01 M, and that of Na_2EDTA in the zonal rotor gradient lowered from 10^{-4} to 2×10^{-5} M. This reduced the overall yield of chloroplasts, but those isolated were more active in protein synthesis than were chloroplasts prepared with the original Mg^{2+} and EDTA concentrations used by Preston *et al.* All buffers used in chloroplast isolation were sterilized by Millipore filtration. Sucrose solutions used to make the zonal rotor gradient were autoclaved; EDTA sterilized by filtration was added to these before use. All glassware was autoclaved, and the French press and zonal rotor parts were cleaned with ethanol before every use. The final chloroplast suspension contained fewer than 100 colony-forming bacteria/ml, among approximately 2×10^9 chloroplasts. When samples of these bacteria were added to the mix normally used for chloroplast protein synthesis, at least 10^4 bacteria/ml were required to equal 10% of the amino acid incorporation exhibited by chloroplast preparations during the first hour of incubation.

Incorporation of Amino Acids. The incorporation mix, a modification of that published by Spencer and Wildman (1964) contained the following components per ml: KCl, 27.6 μmol; Tris-HCl (pH 7.8), 8.6 μmol; magnesium acetate, 20.0 μmol; 2-mercaptoethanol, 3.0 μmol; ATP, 0.4 μmol; GTP, 0.02 μmol; phosphoenolpyruvate, 2.4 μmol; pyruvate kinase, 0.6 EU; sucrose, 0.1 g; ^{14}C-labeled reconstituted protein hydrolysate (Schwarz 3122-08, containing 13 amino acids at an average specific activity of 166 Ci/mol), 4 μCi; and chloroplasts equivalent to 0.8–1.0 mg of chlorophyll.

The reaction was carried out at 25°. Each reaction tube contained a final volume of 0.25 ml, from which four 0.05-ml samples were removed to filter-paper disks at appropriate intervals. These disks were processed by the method of Mans and Novelli (1961), with the washing in ether–ethanol expanded to three 10-min washes in order to remove chlorophyll entirely.

When proteins were labeled for subsequent fractionation,

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[1] Abbreviations used are: RuDPC, ribulose-1,5-diphosphate carboxylase (EC 4.1.1.39); STM buffer, TrisHCl (pH 7.8) 0.01 M, $MgCl_2$ 0.01 M, 2-mercaptoethanol 0.001 M, sucrose 10% (w/v), *cf.* Preston *et al.*, 1972.

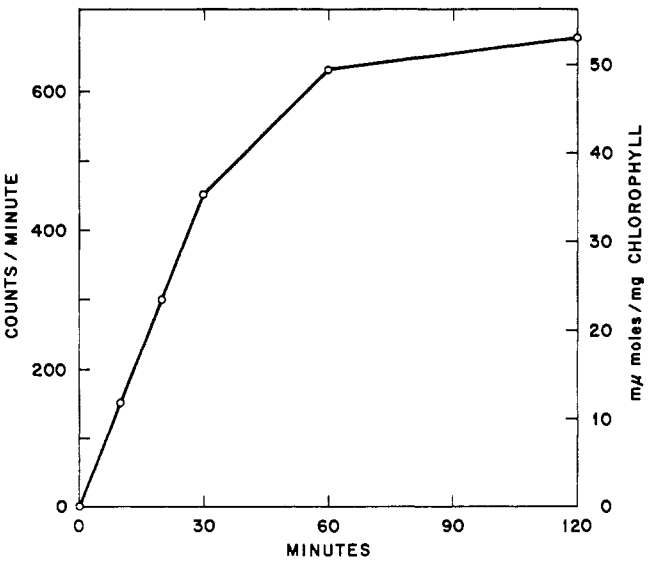

FIGURE 1: Incorporation of amino acids by isolated *Euglena* chloroplasts. Activity is shown on left axis in cpm/0.05 ml of assay, and on right axis in nmol of amino acids incorporated per mg of chlorophyll.

TABLE I: Energy Sources for Chloroplast Protein Synthesis.

Conditions	Amino Acid Incorporation at 40 min: nmol/mg of Chlorophyll	Amino Acid Incorporation at 40 min: % of Control
Control (light)[a]	12.4	100
Control (dark)	12.9	104
−ATP[b]	2.6	21
+ADP, inorganic phosphate, NADP (light)[c]	9.0	73
+ADP, inorganic phosphate, NADP (dark)	5.3	43

[a] Control: system as described in text. [b] −ATP: ATP, phosphoenolpyruvate, and pyruvate kinase omitted from reaction mix. [c] +ADP, inorganic phosphate, NADP: ATP, phosphoenolpyruvate, and kinase omitted, but replaced with ADP, 4.2 μg/ml, K_2HPO_4, 4.0 μg/ml, and NADP, 8.0 μg/ml. "Light" samples were illuminated throughout the reaction with 300 ft-candles of cool white light. "Dark" samples were carefully wrapped with aluminum foil to exclude all light.

the amino acid content of the incorporation mixture was raised to 20 μCi/ml, at 166 Ci/mol, and 2 μg/ml of cycloheximide was added to inhibit incorporation by any contaminating cytoplasmic ribosomes. Reaction mixtures containing 10 ml were incubated for 1 hr at 25° with gentle intermittent shaking. After incorporation the reaction mixture was either disrupted immediately by sonication or stored at −20° until needed.

Incorporation of Nucleoside Triphosphates. RNA synthesis was assayed using isolated chloroplasts equivalent to 1.0–1.5 mg of chlorophyll/ml in a reaction mixture containing, per ml, 0.15 mmol of sucrose; 40 μmol of Tris-HCl (pH 7.8); 12 μmol of 2-mercaptoethanol; 20 μmol of $MgCl_2$; 0.4 μmol each of ATP, GTP, and CTP; 1 mg of Macaloid; and 10 μCi of [^{3}H]UTP (specific activity 19.8 Ci/mmol).

Preparation of Soluble and Lamellar Proteins. Chloroplasts were disrupted by 3 min of sonication in 1-min pulses separated by cooling in ice. Longer sonication released more radioactivity into the soluble protein fraction but appeared to produce some denaturation of proteins. The sonicated suspension was centrifuged for 30 min at 10,000*g*, and the resulting supernatant material was centrifuged for 3 hr at 100,000*g*. The 100,000*g* supernatant liquid is designated S-100 (Figure 4). The material pelleted at 10,000*g* was washed, extracted with acetone, and processed according to the method of Criddle (1966) as used by Goffeau (1969) for the preparation of chloroplast structural protein, and is operationally defined here as the "lamellar protein" fraction.

Column Chromatography. Sephadex G-200 (Pharmacia) was prepared as a column 2.5 × 34 cm in dialysis buffer (Tris-HCl, pH 7.8 0.02 M; $MgCl_2$ 0.001 M; 2-mercaptoethanol, 0.001 M). DEAE-cellulose was prepared in Tris-HCl (pH 7.8 0.05 M) and eluted with a gradient of 0.0–0.6 M NaCl in the same buffer, after the method of Smillie (1968).

Column fractions were sampled for radioactivity by pipetting 0.1 ml onto filter paper disks which were processed by the method of Mans and Novelli (1961). Larger aliquots of column fractions were precipitated with trichloroacetic acid in the presence of bovine serum albumin as carrier protein, heated to 80° for 20 min, and collected on Millipore filter disks. All disks were counted by liquid scintillation in a toluene mix.

Assay for Ribulose Diphosphate Carboxylase. The reaction mixture contained the following components per ml: ribulose diphosphate, 0.375 μmol; [^{14}C]$NaHCO_3$, 18 μCi (specific activity 0.36 Ci/mol); Tris-HCl, pH 7.8, 0.5 mmol; $MgCl_2$, 0.05 mmol; Na_2EDTA, 2.5 μmol; glutathione, 0.075 mmol; enzyme to be tested. The usual sample size was 0.2 ml. The reaction was stopped after 30 min at 25° by addition of 0.05 ml of 6 N acetic acid. A sample of 0.2 ml was removed to a planchet, dried, and counted. Ribulose diphosphate and spinach ribulose diphosphate carboxylase were commercially prepared.

Acrylamide Gel Electrophoresis. Two gel systems were routinely used. The method of Davis (1964) was followed for ordinary gels, and the method of Shapiro *et al.* (1967) for gels containing sodium dodecyl sulfate. All gels were prepared as cylinders of 0.5 × 10 cm with 1-cm spacer gels. Protein was layered directly on top of the spacer gel. Ordinary gels were run at 4°, with 3 mA/tube constant current. Sodium dodecyl sulfate gels were run at room temperature, at 2.5 or 3 mA per tube. After electrophoresis, gels were scanned at 280 mμ in a Gilford linear transport scanner. Radioactive gels were sliced for counting immediately after scanning, and were not subjected to any fixation procedure. The slices were shaken in individual vials overnight in 0.5 ml of 0.1% sodium dodecyl sulfate. Five milliliters of a toluene-based scintillation fluid containing 33% Triton X-100 (v/v) was then added and shaking was continued until this suspension cleared. Other gels were stained with Coomassie Brilliant Blue according to the method of Chrambach *et al.* (1967) and were scanned at 570 mμ.

Preparation of Chloroplasts Labeled in Vivo. Euglena cells in late logarithmic phase were concentrated 10-fold by centrifugation and resuspension in fresh medium. After 5-min incubation, cycloheximide was added to a final concentration of 1 μg/ml. After 5 more min, a mixture of [^{3}H]amino acids

TABLE II: RNA Synthesis by Chloroplasts of *Euglena gracilis*.

Chloroplast	Incorporation of [^{3}H]UTP	
	pmol of UTP/ml	% of Control
Complete	15.1	100
−ATP	4.9	33
−CTP, GTP	6.4	42
−ATP, CTP, GTP	0.2	1
+Actinomycin D (15 μg/ml)	4.1	27
+Rifampicin (5 μg/ml)	5.4	36
+RNase (5 μg/ml)	0.3	2
+DNase (5 μg/ml)	1.0	7

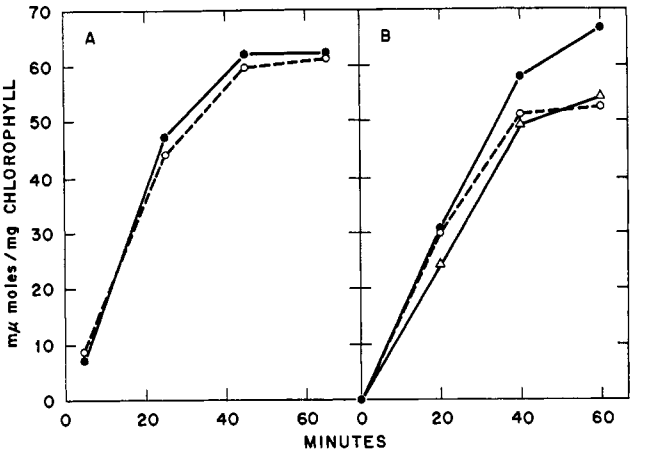

FIGURE 2: Chloroplast protein synthesis. (A) In presence of nucleoside triphosphates needed for RNA synthesis. (●) Control; (○) ATP, CTP, GTP, and UTP added at 0.4 μmol/ml each. (B) In presence of inhibitors of RNA synthesis. (●) Control; (○) actinomycin D, 25 μg/ml; (△) rifampicin, 25 μg/ml.

(Schwarz 3130-07; 16 amino acids at an average specific activity of 7.17 Ci/mol) was added to a final concentration of 4 μCi/ml. The cells were incubated for 90 min in this medium, on a rotary shaker under 180 ft-candles of constant illumination. Chloroplasts were then prepared in the usual manner.

Extraction of Total Protein from Radioactive Chloroplasts. Chloroplast suspensions were made 10% in trichloroacetic acid and the resulting precipitate was collected by centrifugation, washed successively with 10% trichloroacetic acid, ethyl ether–ethanol (3:1) (two times) and ethyl ether (three times), and dried. It was extracted with a buffer containing 0.10 M Tris-HCl (pH 9.0), sodium dodecyl sulfate (1%), and 2-mercaptoethanol (0.14 M). After centrifugation, the supernatant material was dialyzed overnight against the following buffer: 0.01 M sodium phosphate (pH 7.0); 2-mercaptoethanol (0.14 M) and sodium dodecyl sulfate (1%). The final solution was analyzed by acrylamide gel electrophoresis.

Chlorophyll Content and Analysis of Data. Chlorophyll concentration was determined by extraction with 80% acetone (Kirk, 1970). When direct comparison of data from more than one experiment was necessary, all data were expressed in terms of nanomoles of amino acid incorporated per milligram of chlorophyll. Chloroplasts from photoheterotrophically grown cells in early stationary phase contain 1.6 to 1.8 mg of chlorophyll/mg of protein.

Results

Amino acid incorporation by isolated *Euglena* chloroplasts at 25° is linear for 30–45 min and reaches a plateau by 60 min (Figure 1). At 30° the initial incorporation rate is more rapid, but the plateau is reached earlier and total incorporation is slightly less. Incorporation is greatest at pH 7.8 and 0.020 M Mg^{2+}. This high magnesium requirement may result from EDTA added to prevent chloroplast clumping in the zonal rotor. Addition of the seven amino acids not present in the radioactive mix was not necessary for full activity, suggesting that a substantial pool of amino acids is retained by the chloroplasts.

Chloroplast protein synthesis can be supported either by added ATP and an ATP-generating system or by photosynthetic phosphorylation (Table I). A requirement for added ATP has been used as a criterion for distinguishing organelle protein synthesis from that of contaminants (Ranalletti *et al.*, 1969).

Concomitant RNA synthesis may not be necessary for protein synthesis in isolated *Euglena* chloroplasts (Figure 2). Addition of the nucleoside triphosphates needed for RNA synthesis does not appreciably affect the level of incorporation of amino acids, and two drugs known to inhibit RNA synthesis, actinomycin D (Kahan *et al.*, 1963) and rifampicin (Weisblum and Davies, 1968) are ineffective in inhibiting protein synthesis in these chloroplasts. Yet these same isolated chloroplasts actively incorporate [^{3}H]UTP in the presence of ATP, GTP, and CTP, and this incorporation is sensitive both to actinomycin D and to rifampicin (Table II). Thus RNA needed for protein synthesis has apparently been synthesized prior to the beginning of amino acid incorporation, and RNA made by isolated chloroplasts is not utilized immediately in protein synthesis.

Amino acid incorporation by isolated chloroplasts is inhibited by chloramphenicol, tetracycline, spectinomycin, and puromycin (Table III), all of which are known to inhibit protein synthesis in bacteria (Weisblum and Davies, 1968). Cycloheximide, a potent inhibitor of cytoplasmic ribosomes of eucaryotic cells (Siegel and Sisler, 1965), has very little effect, even in extremely high concentrations (10 μg/ml effectively abolishes cytoplasmic protein synthesis in *Euglena* cells). The combination of cycloheximide and chloramphenicol is no more inhibitory than chloramphenicol alone. This suggests that the observed inhibition by high concentrations of cycloheximide does not reflect contamination by cytoplasmic ribosomes, but must represent some other interference with incorporation.

Oligomycin and ouabain, both of which are known to interfere with certain kinds of ATPase activity (Huijing and Slater, 1961; Bonting *et al.*, 1961), were tested in an effort to increase amino acid incorporation by inhibiting destruction of ATP. Although stimulation of mitochondrial protein synthesis by oligomycin has been reported by Lamb *et al.* (1968), neither drug was found to stimulate protein synthesis by *Euglena* chloroplasts.

Chloroplasts from cells grown in medium containing one-tenth the usual concentration of phosphate were also tested in protein synthesis reactions. Microscopic observations (J. F. Preston, unpublished) suggest that chloroplasts from such cells retain greater outer membrane integrity after isolation than do chloroplasts from cells grown in normal medium.

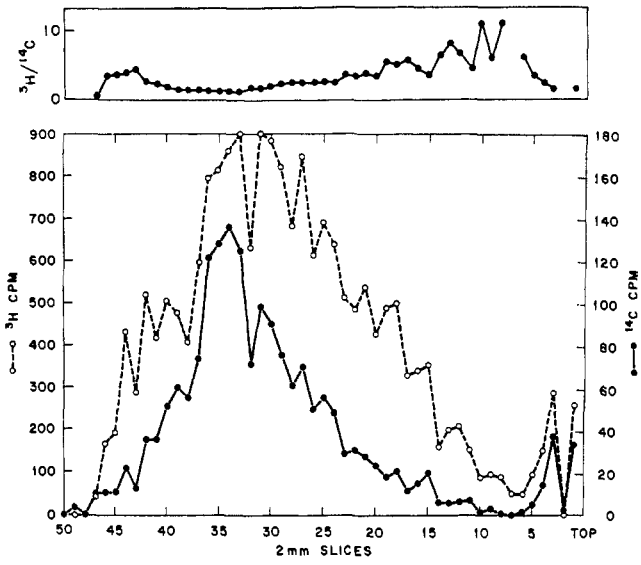

FIGURE 3: Incorporation of radioactive amino acids into chloroplast proteins *in vivo* and *in vitro*. Radioactivity in slices of an acrylamide gel after electrophoresis of a mixture of proteins labeled with [^{3}H]amino acids *in vivo* (○) and proteins labeled in isolated chloroplasts with [^{14}C]amino acids (●). Both protein preparations were obtained by total precipitation with CCl_3COOH and solubilization in alkaline sodium dodecyl sulfate–mercaptoethanol. Slice 12 corresponds to approximately 120,000 daltons; slice 20 to 75,000 daltons; the peaks at slices 28–32 and 32–27 estimated to correspond to 35,000 and 25,000 daltons, respectively. Electrophoresis was at 2.5 ma for 17 hr; gel contained sodium dodecyl sulfate and 7.5% acrylamide. Top of gel at right.

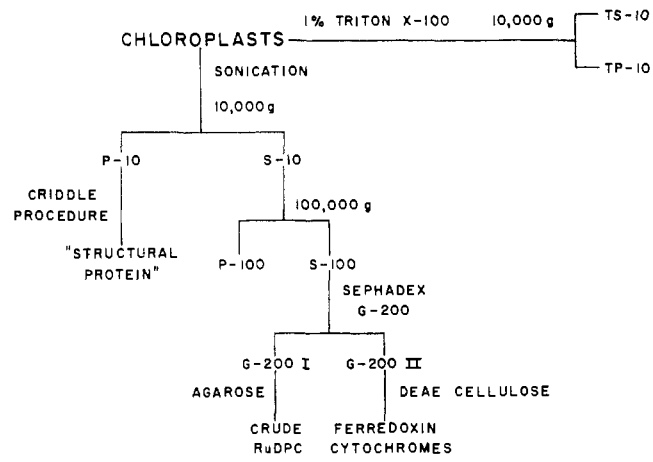

FIGURE 4: Fractionation scheme for preparation of chloroplast proteins. S-10, S-100, and TS-10 are supernatant fractions following centrifugation at indicated *g* force, and P-10, P-100, and TP-10 the respective pellets. "Structural protein" refers to the solubilized lamellar protein prepared by the procedure of Criddle (1966). G-200 I and II are fractions from Sephadex G-200 column as indicated in Figure 6.

Low phosphate chloroplasts are less affected by all the antibiotics tested and consistently show about 70% resistance to inhibition with RNase, which can be reduced by addition of 0.1% Triton X-100 to the reaction mixture.

Total protein precipitation with trichloroacetic acid followed by detergent solubilization and reduction (see Methods) was used to prepare proteins from chloroplasts labeled *in vitro* and *in vivo* in the presence of cycloheximide. These proteins were analyzed and compared by gel electrophoresis in the presence of sodium dodecyl sulfate (Figure 3). Gel slices were counted differentially and the numbers obtained corrected for spillover, using singly labeled gel slices as standards. Molecular weights were estimated from standard marker proteins run on parallel gels.

TABLE III: Effect of Antibiotics and Nucleases on Chloroplast Protein Synthesis.

Conditions	Amino Acid Incorporation at 40 min: nmol/μg of Chlorophyll	Amino Acid Incorporation at 40 min: % of Control
Control	42.0	100
Chloramphenicol, 100 μg/ml	9.2	22
Cycloheximide, 100 μg/ml	34.9	83
Chloramphenicol, 100 μg/ml, + cycloheximide, 100 μg/ml	8.8	21
Puromycin, 20 μg/ml	6.7	16
Tetracycline, 200 μg/ml	13.4	32
Spectinomycin, 72 μg/ml	11.3	27
Oligomycin, 8×10^{-6} M	41.2	98
Ouabain, 8×10^{-3} M	37.4	89
RNase, 40 μg/ml	10.5	25
DNase, 80 μg/ml	37.8	90

In general, the ^{3}H-labeled material synthesized *in vivo* has a greater proportion of components of high molecular weight (right side of figure). Although several peaks labeled *in vivo* are absent or poorly defined in the ^{14}C gel pattern (*in vitro*), there is no prominent peak which is present *only* in the material labeled *in vitro*. Within the region of most extensive labeling there is good correspondence between the two profiles. Thus the products of protein synthesis in isolated chloroplasts can be identified with proteins made on chloroplast ribosomes *in vivo*.

Radioactive material from chloroplasts labeled *in vitro* was separated into soluble and lamellar fractions (Figure 4). In the absence of detergents, the sonication and centrifugation procedures described in the Methods section generate a dark green pellet P-10, a greenish brown pellet P-100, and a pink supernatant fluid S-100. P-10 was used as a source of "structural protein" (see below). P-100 is smaller in volume than P-10 and contains little incorporated radioactivity. If this pellet contains chloroplast ribosomes as expected, no newly synthesized ribosomal proteins are evident. The visible absorption spectrum of S-100 indicates that it contains the chloroplast cytochrome 552 and ferredoxin but little chlorophyll or carotenoid. Approximately 20% of total radioactivity incorporated appears in the S-100 fraction and roughly 75% remains in the P-10 pellet. Triton X-100 releases virtually all incorporated radioactivity into the 10,000*g* supernatant TS-10 (Figure 4).

In gels of dialyzed S-100, the most prominent peaks of radioactivity are fractions of less than 20,000 molecular weight (Figure 5). Of the larger proteins only peak 1 is clearly defined in the radioactive profile. However, the entire range from this peak through peak 10–11 contains radioactivity clearly above background. It appears that a number of different proteins are being labeled, but that most are not being produced in amounts sufficient to constitute a sharp peak. Peak

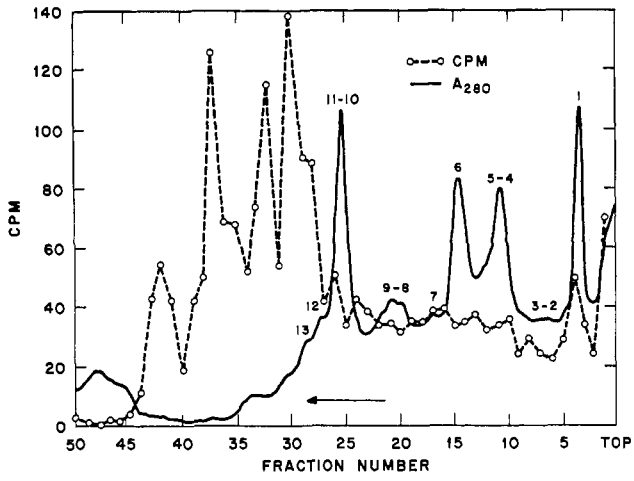

FIGURE 5: Radioactivity of sliced 7% acrylamide gel after electrophoresis of dialyzed soluble proteins (S-100). Continuous solid line indicates absorbance at 280 mμ; points are radioactivity in 2-mm slices of the gel. Numbers indicate 13 protein bands consistently seen in gels of soluble chloroplast proteins.

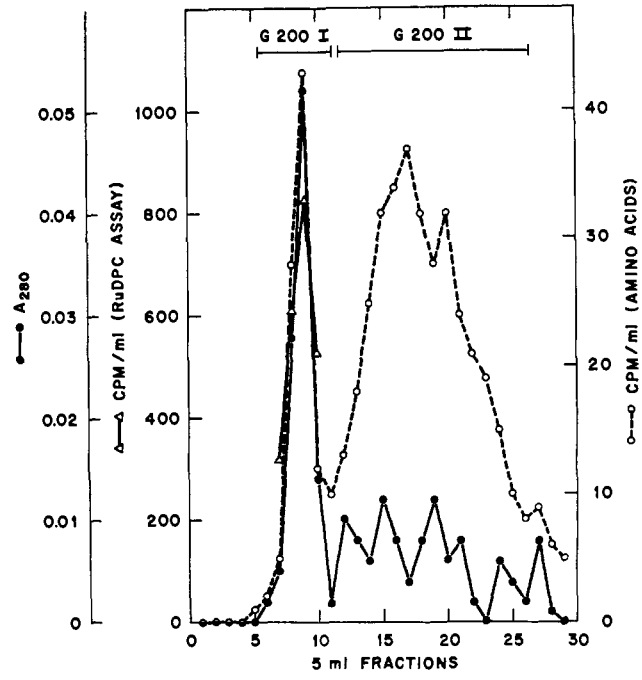

FIGURE 6: Fractionation of soluble chloroplast protein (S-100) on column of Sephadex G-200. (●) Absorbance at 280 mμ; (○) radioactivity from amino acid incorporation, in cpm/ml. (△) Radioactivity (cpm/ml) fixed in assay of 0.2-ml aliquots of fractions for ribulose diphosphate carboxylase (RuDPC). All fractions were tested, but enzyme activity was found only in the fractions indicated.

4–5 represents material of about 60,000 daltons; peak 6 is about 40,000 daltons; and peak 10–11 is about 20,000 daltons. Spinach ribulose diphosphate carboxylase (about 550,000 daltons) migrates very slightly ahead of peak 1 under these electrophoretic conditions.

G-200 I and II are prepared from S-100 on a column of Sephadex G-200 (Figure 6) and represent 25 and 75% of the incorporated radioactivity of S-100 respectively. Ribulose diphosphate carboxylase activity is confined to G-200 I and comigrates with a peak of incorporated radioactivity on a subsequent passage of G-200 I through an agarose column (Figure 7). Protein from Agarose II is coincident with peak 1 of Figure 5 in gel electrophoresis.

G-200 II retains the visible absorption spectrum of S-100, indicating the probable presence of cytochrome(s) and ferredoxin. Radioactive G-200 II was mixed with unlabeled carrier protein enriched in ferredoxin and analyzed on a column of DEAE-cellulose by the method of Smillie (1968). Fractions collected were checked for absorbance at 280, 420, and 552 mμ, and assayed for radioactivity before being pooled into 10 larger fractions (Figure 8). Fraction V contains ferredoxin but has no radioactivity above background; it is concluded that ferredoxin is not made in detectable quantities under these conditions. Fraction I contains virtually all the radioactive material recovered from the column and appears to contain one or more cytochromes, including the soluble chloroplast cytochrome *c* 552. When this fraction was concentrated and analyzed on acrylamide gels, only slight radioactivity above background was found in the upper and central regions of the gel and no radioactive peak occurred near the expected position of cytochrome *c* 552.

Accumulated P-10 pellets were suspended in dialysis buffer, pooled, and washed by centrifugation and resuspension before being treated according to the method of Criddle (1966) for preparation of chloroplast structural protein. The final preparation comprised three ammonium sulfate cuts, which were dissolved in 0.1% sodium dodecyl sulfate and 0.5 M urea and analyzed on acrylamide gels containing sodium dodecyl sulfate. The fraction precipitated at 20–100% ammonium sulfate contains little radioactivity and no prominent peaks of absorption at 280 mμ. Profiles of the 0–10% and 10–20% ammonium sulfate fractions (Figure 9) are suggestive of a heterogeneous population of proteins, many of which are being synthesized in the isolated chloroplasts. The 10–20% ammonium sulfate cut would be expected to contain the principal component of structural protein as prepared from spinach by Criddle (1966). The most prominent absorption peak in this gel is approximately 18,000–20,000 daltons. The smaller peak to the left is estimated at 12,000 daltons. Although the radioactive patterns follow the absorption at 280 mμ in general outline in both fractions, discrete peaks are not well defined and no single "structural protein" can be identified. Insofar as these fractions do represent the insoluble lamellar material from chloroplasts, it may be concluded that a significant fraction of this material is synthesized by isolated chloroplasts.

In other experiments, protein labeled with [^{3}H]amino acids *in vivo* in the presence of cycloheximide was prepared and fractionated as described above. The patterns of radioactivity in S-100, G-200 I, and G-200 II, and lamellar proteins on gel electrophoresis closely resemble those obtained with protein labeled *in vitro*. This confirms the conclusion drawn from the experiment shown in Figure 3, that protein synthesis in isolated chloroplasts does not differ greatly from the pattern of synthesis on chloroplast ribosomes *in vivo*.

Discussion

In many respects, this chloroplast system for protein synthesis resembles others from *Euglena* (Eisenstadt and Brawerman, 1964; Gnanam and Kahn, 1967; Reger *et al.*, 1972) and from higher plants (*e.g.*, Bamji and Jagendorf, 1966; Ellis, 1970; Parenti and Margulies, 1967; Ranalletti *et al.*, 1969; Spencer and Wildman, 1964). However, chloroplasts isolated by our method appear to retain an unusually high degree of physiological integrity, which is significant in that

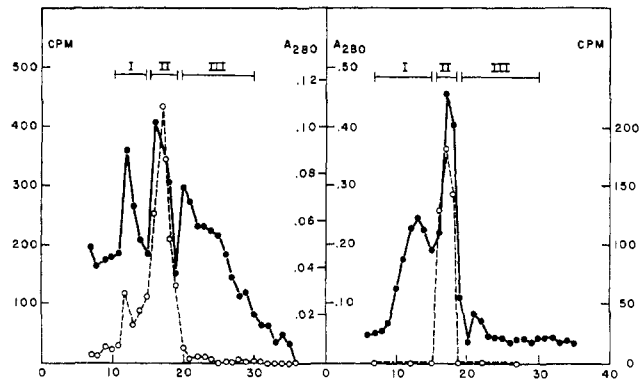

FIGURE 7: Fractionation of G-200 I on agarose column. Left: Radioactive G-200 I from chloroplasts labeled with amino acids *in vitro*. (●) A_{280}; (○) cpm in 3-ml samples from 200-drop fractions. Right: RuDPC activity in unlabeled G-200 I. (●) A_{280}; (○) cpm of 0.2 ml of RuDPC assays, using 0.1-ml samples from 200-drop fractions.

it permits experiments difficult or impossible with the other systems.

Our observation that chloroplast protein synthesis does not require concomitant RNA synthesis contrasts sharply with the results of Chi and Suyama (1970), who found that addition of nucleoside triphosphates greatly stimulated amino acid incorporation by *Tetrahymena* mitochondria. Likewise, actinomycin D and rifampicin were found not to inhibit chloroplast protein synthesis before 40 min of incorporation. This implies that protein synthesis in *Euglena* chloroplasts is not necessarily coupled to continuous production of template RNA and is consistent with the interesting possibility that chloroplast ribosomes might translate mRNA previously transcribed from a *nuclear* DNA template.

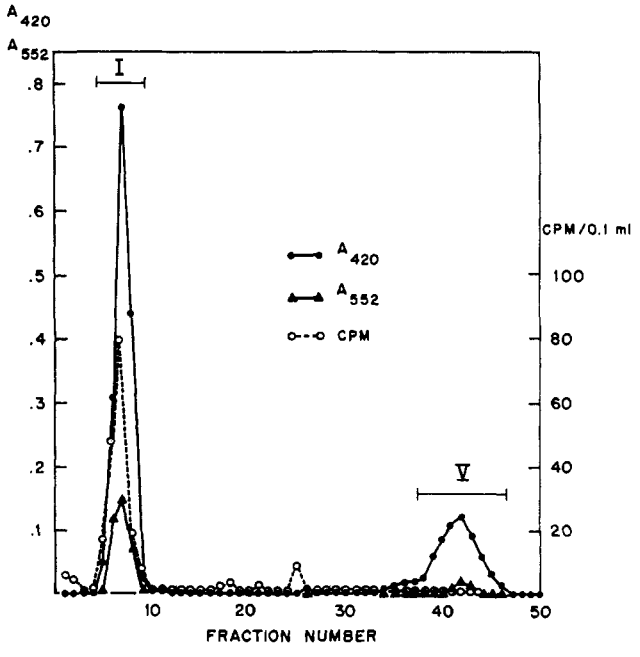

FIGURE 8: Chromatography of G-200 II fraction of soluble proteins on DEAE-cellulose. (●) Absorbance at 420 mμ; (▲) absorbance at 552 mμ; (○) radioactivity in 0.1-ml aliquots of 3.5-ml fractions. Elution was with 0–0.6 M NaCl gradient. Roman numerals indicate fractions which were analyzed further: I contains one or more cytochromes; V, which was eluted at 0.2–0.3 M NaCl, contains ferredoxin.

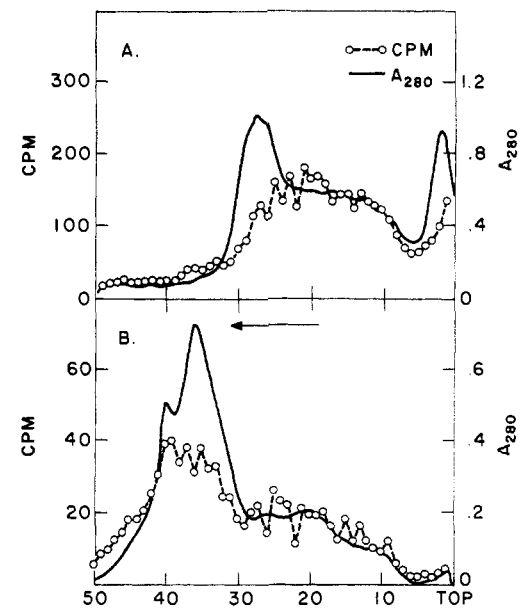

FIGURE 9: Electrophoresis of lamellar proteins in 10% acrylamide gel containing sodium dodecyl sulfate. (A) Sample was 0–10% ammonium sulfate cut of solubilized lamellar proteins. Continuous line is absorbance at 280 mμ, and points are radioactivity in 2-mm slices. Electrophoresis for 16 hr at 3 mA. (B) Gel as in part A, but with 10–20% ammonium sulfate cut of lamellar proteins.

Analysis of chloroplast ribosomes in sucrose gradients indicates that incorporation of amino acids during the first five minutes of incubation is associated with large polysomes, which decrease in size as protein synthesis progresses (Harris and Eisenstadt, 1971). Thus polysome formation in isolated chloroplasts does not appear to require concomitant synthesis of RNA and is similar to that previously described for mammalian cells (Lee *et al.*, 1971).

Analysis of protein fractions after amino acid incorporation indicates that a variety of proteins are synthesized on chloroplast ribosomes, both in isolated chloroplasts and in whole cells in which cytoplasmic ribosomes have been inhibited by cycloheximide treatment. The products of one hour of amino acid incorporation *in vitro* range in size from polypeptides of less than 10,000 daltons to at least one very large protein with a molecular weight around 600,000 daltons. Prominent among these products are lamellar proteins of 15,000 to 40,000 daltons which can be extracted from chlorophyll-containing particulate matter only by solubilization with detergents.

The distribution of incorporation into soluble and lamellar proteins reported here resembles results with chloroplasts from other sources (Margulies and Parenti, 1968; Goffeau, 1969; Chen and Wildman, 1970; Spencer *et al.*, 1971). Only 20–30% of the incorporated radioactivity appears in proteins extractable with water, but the specific activities of the soluble and lamellar proteins are very nearly the same. The solubilization of incorporated radioactivity by treatment with Triton X-100 is similar to that reported with bean chloroplasts by Parenti and Margulies (1967) and differs from the results of Goffeau (1969), who found that Triton treatment did not release lamellar incorporation in *Acetabularia* chloroplasts.

Synthesis of lamellar proteins, in particular the "structural protein" operationally defined by the preparation method of Criddle (1966), extends to *Euglena* the observation that such

proteins are indeed products of chloroplast ribosomes, as reported in chloroplasts isolated from bean leaves (Margulies and Parenti, 1968) and from *Acetabularia* (Goffeau, 1969). Our results suggest that much of the protein material solubilizable with sodium dodecyl sulfate is labeled during chloroplast protein synthesis *in vitro*. This implies a high degree of organelle autonomy for the translation of chloroplast structural components.

Our results also suggest the translation of a number of soluble proteins in isolated chloroplasts, among them "Fraction I protein" (crude ribulose diphosphate carboxylase), the synthesis of which has also been reported in isolated bean chloroplasts (Margulies and Parenti, 1968) and pea chloroplasts (Blair and Ellis, 1972). Further experiments are in progress to establish whether labeled amino acids have been incorporated into the structure of the purified enzyme. In higher plants and in *Chlamydomonas*, increase in this enzyme activity is sensitive to cycloheximide (Armstrong *et al.*, 1971; Chen *et al.*, 1967; Keller and Huffaker, 1967) and it has been suggested that synthesis of the small subunit may occur on cytoplasmic ribosomes (Criddle *et al.*, 1970). Since Smillie *et al.* (1967) have found that cycloheximide does not prevent increase of ribulose diphosphate carboxylase activity in *Euglena in vivo*, an unequivocal demonstration of synthesis of this enzyme in isolated *Euglena* chloroplasts would confirm that *Euglena* differs from other species in this respect.

Smillie *et al.* (1967) also found that synthesis of the chloroplast cytochrome 552 is prevented by both chloramphenicol and cycloheximide in greening *Euglena*. They concluded that this protein is probably made on chloroplast ribosomes but that its synthesis also requires some structural component for which protein synthesis on cytoplasmic ribosomes is necessary. Our evidence that lamellar proteins are abundantly synthesized in isolated chloroplasts, in conjunction with the failure to detect obvious labeling after gel electrophoresis which might correspond to cytochrome *c* 552, suggests that the converse may be true.

Partial purification of ferredoxin indicates that this protein is not being synthesized at a detectable level under the conditions used here. This result contrasts with those of Spencer *et al.* (1971), who have presented evidence for synthesis of ferredoxin in isolated spinach chloroplasts, and with those of Gibbons *et al.* (1969), who have reported that ferredoxin is a product of chloroplast ribosomes in *Euglena*, based on antibiotic studies *in vivo*.

In dense *Euglena* cultures, chlorophyll and galactolipids increase in a constant proportion after cell division has declined, while system I electron-transport proteins remain constant (Melandri *et al.*, 1970; Böger and San Pietro, 1967). This suggests that the final stages of chloroplast development may not involve synthesis of ferredoxin, cytochrome 552 or NADP-reductase. All the experiments reported here have been conducted with chloroplasts from mixotrophically grown cells in early stationary phase. In preliminary experiments such cells produced the best yields of chloroplasts with substantial activity in synthesis of RNA and protein. Since these cells are undergoing a final stage in photosynthetic development as their culture medium becomes exhausted and cell division stops, it is quite possible that certain chloroplast components are already present in adequate supply. Synthesis of such components could be repressed at this stage, whereas it would normally occur in chloroplasts of dividing and/or greening cells. In any case, we conclude that *Euglena* chloroplasts do have an appreciable measure of autonomy in the translation of their structural and soluble proteins.

References

Armstrong, J. J., Surzycki, S. J., Moll, B., and Levine, R. P. (1971), *Biochemistry 10*, 692.

Bamji, M. S., and Jagendorf, A. T. (1966), *Plant Physiol. 41*, 764.

Blair, G. E., and Ellis, R. J. (1972), *Biochem. J. 127*, 42P.

Böger, P., and San Pietro, A. (1967), *Z. Pflanzenphysiol. 58*, 70.

Bonting, S. L., Simon, K. A., and Hawkins, N. M. (1961), *Arch. Biochem. Biophys. 95*, 416.

Chen, J., and Wildman, S. G. (1970), *Biochim. Biophys. Acta 209*, 207.

Chen, S., McMahon, D., and Bogorad, L. (1967), *Plant Physiol. 42*, 1.

Chi, J. C. H., and Suyama, Y. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol.* 3518 Abstr.

Chrambach, A., Reisfeld, R. A., Wyckoff, M., and Zaccari, J. (1967), *Anal. Biochem. 20*, 150.

Criddle, R. S. (1966), *in* Biochemistry of Chloroplasts I, Goodwin, T. W., Ed., London, Academic Press, pp 203–231.

Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.

Eisenstadt, J. M., and Brawerman, G. (1964), *J. Mol. Biol. 10*, 392.

Ellis, R. J. (1970), *Planta 91*, 329.

Gibbons, G. C., Raison, J. K., and Smillie, R. M. (1969), *Proc. Australian Biochem. Soc. 2*, 43.

Gnanam, A., and Kahn, J. S. (1967), *Biochim. Biophys. Acta 142*, 486.

Goffeau, A. (1969), *Biochim. Biophys. Acta 174*, 340.

Harris, E. H., and Eisenstadt, J. M. (1971), *Biochim. Biophys. Acta 232*, 167.

Huijing, F., and Slater, E. C. (1961), *J. Biochem. (Tokyo) 49*, 493.

Kahan, E., Kahan, F. M., and Hurwitz, J. (1963), *J. Biol. Chem. 238*, 2491.

Keller, C. J., and Huffaker, R. C. (1967), *Plant. Physiol. 42*, 1277.

Kirk, J. T. O. (1970), *Annu. Rev. Plant Physiol. 21*, 11.

Lamb, A. J., Clark-Walker, G. C., and Linnane, A. W. (1968), *Biochim. Biophys. Acta 161*, 415.

Lee, S. Y., Krsmanovic, K., and Brawerman, G. (1971), *Biochemistry 10*, 895.

Mans, R. J., and Novelli, G. D. (1961), *Arch. Biochem. Biophys. 94*, 48.

Margulies, M. M. (1970), *Plant Physiol. 46*, 136.

Margulies, M. M., and Parenti, F. (1968), *Plant Physiol. 43*, 504.

Melandri, B. A., Baccarini-Melandri, A., and San Pietro, A. (1970), *Arch. Biochem. Biophys. 138*, 598.

Parenti, F., and Margulies, M. M. (1967), *Plant Physiol. 42*, 1179.

Preston, J. F., Parenti, F., and Eisenstadt, J. M. (1972), *Planta 107*, 351.

Ranalletti, M., Gnanam, A., and Jagendrof, A. T. (1969), *Biochim. Biophys. Acta 186*, 192.

Reger, B. J., Smillie, R. M., and Fuller, R. C. (1972), *Plant Physiol. 50*, 24.

Shapiro, A. L., Viñuela, E., and Maizel, J. V. (1967), *Biochem. Biophys. Res. Commun. 28*, 815.

Siegel, M. R., and Sisler, H. D. (1965), *Biochim. Biophys. Acta 103*, 558.

Smillie, R. M. (1968), *in* The Biology of Euglena, Buetow, D. E., Ed., New York, N. Y., Academic Press, pp 2–54.

Smillie, R. M., Graham, D., Dwyer, M. R., Grieve, A., and Tobin, N. F. (1967), *Biochem. Biophys. Res. Commun. 28*, 604.

Spencer, D., Whitfield, P. R., Bottomley, W., and Wheeler, A. M. (1971), *in* Autonomy and Biogenesis of Mitochondria and Chloroplasts, Boardman, N. K., Linnane, A. W., and Smillie, R. M. Ed., Amsterdam, North-Holland, pp 372–382.

Spencer, D., and Wildman, S. G. (1964), *Biochemistry 3*, 954.

Weisblum, B., and Davies, J. (1968), *Bacteriol. Rev. 32*, 493.

Kinetics of Ribonucleic Acid–Deoxyribonucleic Acid Membrane Filter Hybridization†

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ABSTRACT: The kinetics of hybridization of RNA to DNA immobilized on membrane filters were examined. It was found that hybridization binding curves could not be described in terms of a single forward and a single reverse rate constant for the formation and dissociation of the hybrid. Detection of a new form of hybrid provided additional evidence that the adsorption process was more complex. This new hybrid form, although stable to conditions which removed nonspecific hybrid, was more readily dissociated and more sensitive to RNase treatment than the final hybrid form. The amount of this less stable hybrid bound to the filters was inversely related to the amount of stable hybrid bound. Furthermore, the unstable hybrid could be converted into stable RNase-resistant hybrid under hybridization conditions. From these results we conclude that the unstable hybrid is a direct intermediate in hybrid formation and that both the formation and the conversion of the intermediate are rate determining in the kinetics of the overall reaction.

The kinetics and the mechanism of association of single strands of nucleic acid when both strands are in solution have been widely studied (Wetmur and Davidson, 1968; Craig *et al.*, 1971; Porshke and Eigen, 1971). While it has been found that the mechanism of association is complex, intermediates in the reaction do not accumulate, and thus the reaction appears to have only one kinetically significant step.

DNA–RNA hybridization on membrane filters have also been assumed to be closely approximated by the kinetics of a one-step adsorption reaction (Perry *et al.*, 1964; Mangiarotti *et al.*, 1968; Lavallé and De Hauwer, 1968; Kennell, 1968). However, this assumption has not been precisely tested, and the actual mechanism of strand association in membrane filter hybridization remains obscure.

For example, the annealing of RNA to membrane filter bound DNA is generally agreed to reach an apparent steady state after 24 hr (at 66° and 0.33 M sodium). However, Bishop (1970) has shown that the dissociation constant measured after 24 hr does not equal either the equilibrium constant calculated from the ratio of the reverse to the forward rate constant for the reaction or the equilibrium constant calculated by extrapolating the dissociation constant to infinite time. The simplest explanation for this discrepancy is that, after 24 hr, hybridizations have not reached equilibrium. An alternative explanation is that Bishop's rate constants are distorted by the presence of fast and slow reacting DNA sites. Still another alternative is that this discrepancy reflects a more complex kinetic mechanism for membrane filter hybridizations. To examine these alternatives, we investigated in detail the kinetics of membrane filter hybridizations.

For these investigations we used as a model system the hybridization of bacterial ribosomal RNA (rRNA) to total bacterial DNA. This system offers the advantages of natural polynucleotides with little internal redundancy of sequences (Fellner, 1971). Furthermore, the polynucleotides are easy to isolate in highly pure radioactive form. While there are three distinct ribosomal RNA species in our hybridizations (23S, 16S, and 5S), the RNA species and their DNA sites are present in the ratios of 1:1:1, and thus the hybridization can be considered in terms of a single RNA species (Avery and Midgely, 1969).

Our investigations showed that the kinetics of membrane filter hybridizations were not those expected of a one-step nonequilibrium adsorption process. We found no evidence of fast and slow reacting DNA sites which could explain our results. However, we detected a second, less stable form of DNA–RNA hybrid. The properties of this new form of hybrid suggest that it is a direct kinetic intermediate in the hybridization reaction.

Methods

DNA Isolation. All of the DNA used in these hybridizations was labeled with tritiated thymidine. Cultures of *Bacillus cereus* T were grown in YP medium (4 g/l. of Bacto-peptone (DIFCO), 0.5 g/l. of yeast extract (DIFCO)) containing 5 mCi/l. of [^{3}H]thymidine (New England Nuclear Corp., 6.7 Ci/mmol). DNA (final specific activity of 4.3×10^4 dpm/μg) was isolated from stationary phase cultures by a modification of the method of Marmur (1961) in which redistilled phenol saturated with 0.05 M Tris buffer, pH 7.5, was used for deproteinization.

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