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Nonterminal Incorporation of Guanosine Monophosphate from Guanosine Triphosphate by an Enzyme System from Spinach Chloroplasts*

ARUN K. CHAKRAVORTY AND B. B. BISWAS

From the Radiochemical Laboratory, Bose Institute, Calcutta 9, India

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Ribonucleoside triphosphates can react enzymatically to yield inorganic pyrophosphate and homopolymers of adenosine, cytosine, and uridine monophosphates (1–7). The number of newly incorporated homologous nucleotide residues in each chain varies from 2 to 100 or more. In most cases, the reaction is dependent on the presence of ribonucleic acid or oligoribonucleotide. A purified enzyme from calf thymus nuclei described by Edmonds and Abrams (8) catalyzes the synthesis of polyadenylic acid more than 100 units long. Its activity is dependent upon an oligonucleotide with an adenylate sequence. Another enzyme system isolated either from *Escherichia coli* (9) or from calf thymus nuclei (10) predominantly incorporates ribonucleotides into terminal positions of deoxyribonucleic acid, and the resulting ribonucleotidyl deoxyribonucleic acid can serve as an acceptor for ribonucleotides when incubated with enzymes which normally incorporate ribonucleotides into ribonucleic acid (11, 12). DNA-dependent synthesis of polyadenylic, polyuridylic, and polycytidylic acids has also been demonstrated by a number of investigators (13–17). Polycytidylic acid-dependent incorporation of GMP from GTP by RNA polymerase has recently been described by Nakamoto and Weiss (18) and by others (19, 20). Besides this, the synthesis of polyguanylic acid of limited chain length has been carried out with polynucleotide phosphorylase by Singer, Hilmoe, and Heppel (21) and subsequently by Fresco and Su (22). On the other hand, incorporation of GMP into a polynucleotide chain from GTP in a manner analogous to that reported for other nucleoside triphosphates (4, 6) has not as yet been achieved. It has been recently reported from this laboratory (23) that crude extracts from spinach chloroplasts are capable of catalyzing the incorporation of ribonucleotides into polynucleotide material. The purpose of this communication is to describe the preparation and properties of an enzyme system from spinach chloroplasts which catalyzes the nonterminal incorporation of GMP from GTP into a polynucleotide chain.

EXPERIMENTAL PROCEDURE

Materials

Radioactive nucleoside triphosphates were purchased from Schwarz BioResearch, Inc., and the nonradioactive nucleosides, nucleoside mono-, di-, and triphosphates from Sigma Chemical Company. Actinomycin D was obtained through the courtesy of Merck, Sharp and Dohme Research Laboratories. Carrier-free $H_3{}^{32}PO_4$ was purchased from the Atomic Energy Establishment, Trombay, India. Taka-Diastase was a gift from Dr. Mary Edmonds, Montefiore Hospital, Pittsburgh, Pennsylvania.

Methods

Preparation of Chloroplasts—Chloroplasts were prepared and purified from spinach leaves (*Beta vulgaris*) by the method of Gorham (24).

Isolation of GTP-incorporating Enzyme System—All of the operations were carried out at 0–4°. About 40 g of fresh or frozen chloroplasts were suspended in 10 volumes of glass-distilled water and stirred at top speed with a magnetic stirrer for 60 min followed by centrifugation at 10,000 × *g* for 30 min. By this operation, the chloroplasts were fractionated into an almost colorless stroma (supernatant) and intense green grana (pellet). The enzyme was isolated from the grana fraction by extracting with Tris buffer. This was done by homogenizing the grana with prechilled alumina for about 30 min and finally extracting with 0.1 M Tris, pH 7.2; the volume of buffer used being 6 to 10 times the weight of chloroplasts initially taken. The extract was clarified by centrifugation at 10,000 × *g* for 30 min. The supernatant was brought to 30% saturation by the slow addition of ammonium sulfate (16.4 g/100 ml) with constant stirring. After equilibrating for 30 min, the precipitate was collected by centrifugation at 10,000 × *g* for 30 min and dissolved in 0.05 M Tris, pH 7.2. The resulting solution was dialyzed overnight against 2-liter portions of the same buffer. The preparation contained 35 to 40 μg of RNA per mg of protein and no detectable diphenylamine-reacting material.

Preparation of GT^{32}P—GM^{32}P was prepared from isopropylidine guanosine by the method described by Weiss (25). GM^{32}P was enzymatically converted to GT^{32}P with an extract from ascites tumor cells as reported by Edmonds and Abrams (1). GT^{32}P was isolated by chromatography on Dowex 1-formate (8%, 200 to 400 mesh) with stepwise elution with formic acid and mixtures of formic acid and ammonium formate. The GT^{32}P eluted from the column was desalted by passing through Dowex 50 (H^+), dried in a vacuum, and further purified by paper chromatography on Whatman No. 1 chromatographic paper with an isobutyric acid (100 ml)-1 M NH_4OH (60 ml)-0.2 M EDTA (0.8 ml) solvent system.

Synthesis and Isolation of Poly A,[1] *Poly U, and Poly C*—These

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[1] The abbreviations used are: poly A, polyadenylic acid; poly C, polycytidylic acid; poly G, polyguanylic acid; poly I, polyino-

homopolymers were synthesized by a partially purified (to the calcium phosphate gel and ammonium sulfate step) polynucleotide phosphorylase from *Azotobacter vinelandii* (26). The polymers were deproteinized and purified according to the method of Jones *et al.* (27).

Preparation of DNA and RNA—DNA was isolated from spinach chloroplasts as described earlier by Biswas and Biswas (28). RNA from spinach leaves was obtained from the first supernatants during the preparation of chloroplasts from whole leaf homogenates. After precipitation with alcohol, RNA was redissolved in water, and the solution was extracted with aqueous phenol and further deproteinized by chloroform-octanol (4:1). The final preparation (280:260 absorbance ratio of 0.52) was dialyzed against glass-distilled water before use.

Preparation of RNase T_1—Ribonuclease T_1 was prepared from Taka-Diastase by the method of Takahashi (29).

Assay Method for GMP Incorporation—The incubation mixture contained in a final reaction volume of 1 ml the following ingredients (in micromoles unless otherwise stated): Tris buffer, pH 8.5, 100; $MnCl_2$ 5; GTP-8-^{14}C (specific activity, 5 to 8 $\times 10^5$ cpm per μmole), 0.4; and the enzyme preparation, 1 to 2 mg of protein. The incubation was carried out at 37° for 15 min, and the reaction was terminated by adding 10 ml of cold 1 M perchloric acid. The precipitate, after standing for 20 to 30 min, was centrifuged, washed with cold 0.2 N perchloric acid six times, and dispersed in 0.1 M NH_4OH. This was reprecipitated with cold 1 M perchloric acid, and the precipitate was washed four times as above before it was finally dispersed in 0.1 M NH_4OH. An aliquot was plated for determination of radioactivity in a windowless gas flow counter.

Determination of Terminal and Internal Incorporation—The reaction product (3000 to 5000 cpm) was subjected to alkaline hydrolysis (30) and after adding an adequate quantity (1.5 to 2.0 μmoles) of nonradioactive guanosine and guanosine 2′(3′)-phosphate, these were isolated by chromatography on a Dowex 1-formate column (31), and the radioactivity associated with each was determined (Method 1). In other instances, the hydrolysate was concentrated and 0.5 μmole each of guanosine, guanosine 2′(3′)-phosphate, and guanosine 2′(3′)5-diphosphate was added (nonradioactive). The material was then subjected to one-dimensional descending chromatography on Whatman No. 1 paper with the same solvent system as employed for the purification of GT^{32}P (Method II).

Pyrophosphate Estimation—Pyrophosphate was heated at 100° with 1 N HCl, and the resulting orthophosphate was estimated by the method of Lowry and Lopez (32). The quantity of pyrophosphate released during GMP incorporation was determined as described by Fox *et al.* (20).

Estimation of Nucleic Acids and Protein—Estimation procedures for RNA, DNA, and protein have been described (31).

RESULTS

When spinach chloroplasts are fractionated into grana and stroma, the stroma fraction is found to catalyze the incorporation of UMP and CMP into polynucleotide material. From the grana on the other hand, an enzyme fraction has been isolated which catalyzes the incorporation of AMP and GMP.

sinic acid; poly U, polyuridylic acid; poly AG, copolymer of adenylic and guanylic acids; RNase T_1, ribonuclease from Taka-Diastase.

TABLE I

Incorporation of ribonucleotides by grana enzyme

Incubation conditions and assay procedure are described under "Methods." $MgCl_2$ (5 μmoles per ml) and each of the ribonucleoside triphosphates (0.4 μmole per ml) were used. Specific activities of radioactive ribonucleoside triphosphates (counts per min per μmole) were: ATP-8-^{14}C, 5.4 $\times 10^5$; GTP-8-^{14}C, 5.0 $\times 10^5$; UTP-2-^{14}C, 3.6 $\times 10^5$; CTP-2-^{14}C, 2.7 $\times 10^5$.

Substrate	Incorporation
	mμmole/mg protein
GTP-8-^{14}C	0.31
ATP-8-^{14}C	0.32
GTP-8-^{14}C + ATP	0.19
GTP-8-^{14}C + ATP + UTP + CTP	0.18
GTP-8-^{14}C + ITP	0.17
CTP-2-^{14}C	<0.07
UTP-2-^{14}C	<0.06

TABLE II

Cation requirement for GMP incorporation

Conditions: 5 μmoles per ml of each cation were used; other conditions are as described under "Methods." In the case of Cu^{++}, glycylglycine buffer, pH 8.5, was used.

Cation	Incorporation
	mμmole/mg protein
Mn^{++}	0.69
Mg^{++}	0.34
Cu^{++}	0.32
Ca^{++}	0.32
Cd^{++}	0.27
Pb^{++}	0.20
Zn^{++}	0.16
Na^{+}	0.20
K^{+}	0.22
None	0.14
None + EDTA (10 μmoles)	0.12

It has further been found that incorporation of GMP by this grana enzyme is inhibited by ATP and vice versa.

Table I represents the data pertaining to the incorporation of radioactive nucleotides by the grana extract. It can be seen that the preparation catalyzes the incorporation of either GMP or AMP and the incorporation is optimal when either of these nucleoside triphosphates is present alone in the incubation mixture. Incorporation of GMP is inhibited by ATP and also by a mixture of ATP + UTP + CTP approximately to the same extent. CMP and UMP are not incorporated by the system and the presence of CTP and UTP does not promote or inhibit the incorporation of GMP. It has been found that the incorporation of AMP is influenced exactly in the same manner as that of GMP by either GTP alone or GTP + UTP + CTP (23). Besides ATP, ITP also inhibits the incorporation of GMP.

Cation Requirement—Incorporation of GMP by the enzyme requires a divalent cation for optimal activity. Although Mg^{++} satisfies this requirement, Mn^{++} is twice as active. Monovalent cations such as Na^{+} and K^{+} are largely ineffective; however, there is some incorporation in the presence of Cu^{++} and Ca^{++}. The effects of different cations on the incorporation of GMP are

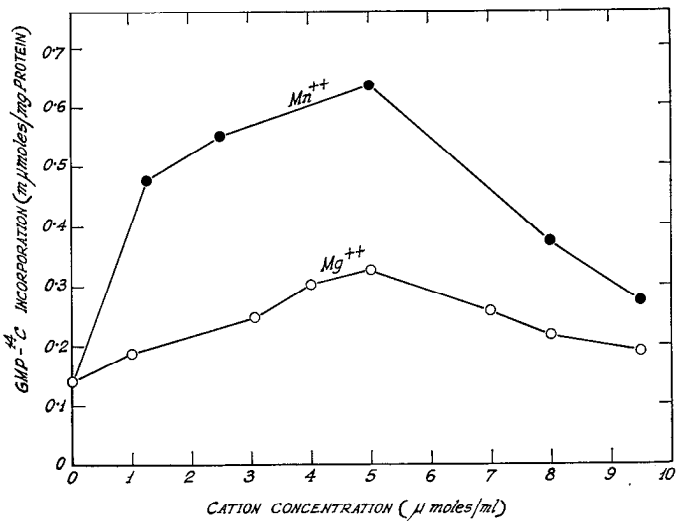

FIG. 1. Incorporation of GMP with different concentrations of Mg^{++} and Mn^{++}. Standard incubation conditions and assay procedure as described under "Methods" have been used.

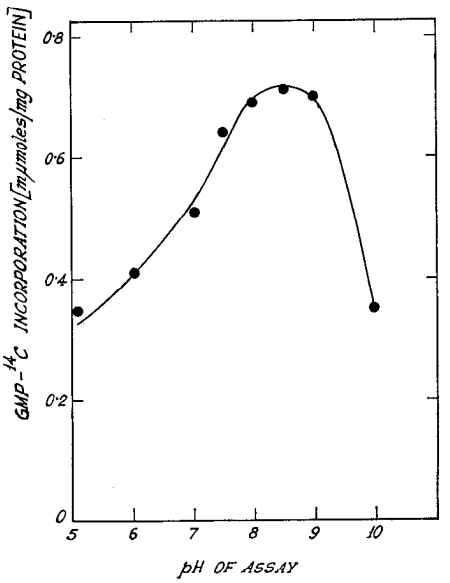

FIG. 2. GMP incorporation with different pH values. The buffers used were: acetic acid-sodium acetate, pH 5.0; succinic acid-NaOH, pH 6.0; Tris maleate-NaOH, pH 7.0; Tris-HCl, 7.5 to 9.0; glycine-NaOH, pH 10.0. Other conditions were as indicated under "Methods."

listed in Table II. Some activity can be observed even in presence of EDTA (10 μmoles per ml) and also without the addition of any cation. Fig. 1 shows the effect of different concentrations of Mg^{++} and Mn^{++} on the incorporation of GMP from GTP. About 5 μmoles per ml of either Mg^{++} or Mn^{++} are required for optimal activity, both being inhibitory beyond this concentration under the standard assay conditions.

pH Optimum—The pH curve (Fig. 2) shows that the optimum for GMP incorporation lies between pH 8 and 9, there being a rapid fall of activity beyond pH 9.

Linearity with Time and Enzyme Concentration—GMP incorporation increases almost linearly with time up to about 10 min after which the rate of incorporation gradually decreases (Fig. 3). Under the given conditions of substrate concentration (0.4 μmole per ml) and temperature (37°), incorporation is also proportional to enzyme concentration up to a limit (1.75 to 2 mg of protein per ml) as shown in Fig. 4.

Effect of Substrate Concentration—The incorporation of GMP is dependent on the concentration of GTP. When the inverse of substrate concentration (micromoles per ml) is plotted against the inverse of reaction rate (millimicromoles of GMP incorporated per mg of protein per 6 min at 37°) the apparent K_m value, as calculated from Fig. 5, is approximately 9×10^{-5} M. The K_i value (4.3×10^{-5} M) for both ATP and ITP seems to be the same, and the inhibition appears to be competitive.

Other Properties—Incorporation of GMP from GTP is accompanied by an equimolar release of pyrophosphate. The value

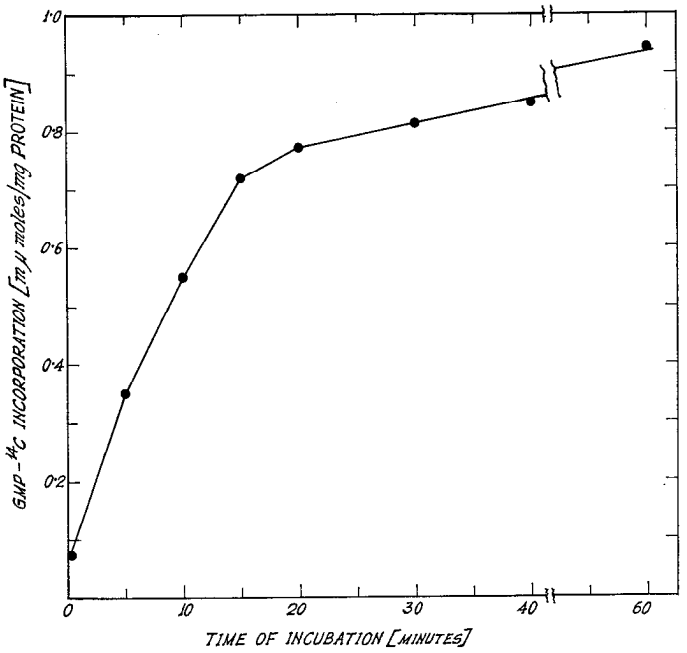

FIG. 3. Incorporation of GMP as a function of time. Incubation conditions and assay procedure as mentioned under "Methods" have been used.

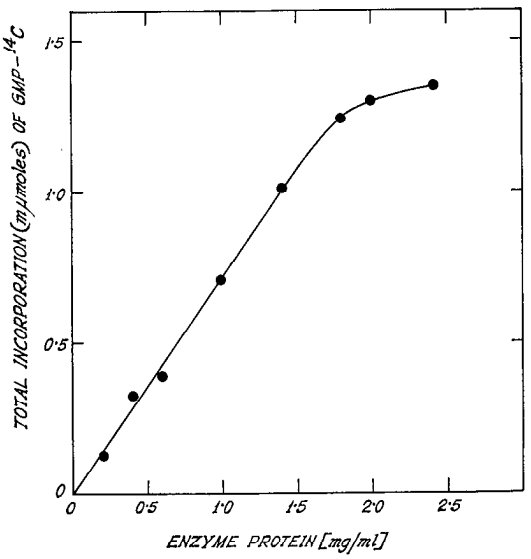

FIG. 4. Incorporation of GMP as a function of enzyme protein concentration. Standard incubation conditions and assay procedure have been used.

of pyrophosphate released (0.65 mμmole per mg of protein) under standard reaction conditions corresponds favorably to the observed value of GMP-^{14}C incorporation under the same conditions. It has also been found that incorporation of GMP is inhibited by pyrophosphate but not by orthophosphate (Table III). Data presented in Table III also indicate that GTP and not GDP or GMP is the actual substrate of the enzyme. The presence of more than twice as high a concentration of GDP or GMP as GTP does not affect the reaction. It is also shown that GM^{32}P is not directly incorporated by the enzyme.

The data presented in Table III thus eliminate the possibility of the involvement of polynucleotide phosphorylase in this reaction since the synthesis of polynucleotides by polynucleotide phosphorylase is inhibited by orthophosphate, and this enzyme specifically requires nucleoside diphosphates as substrates.

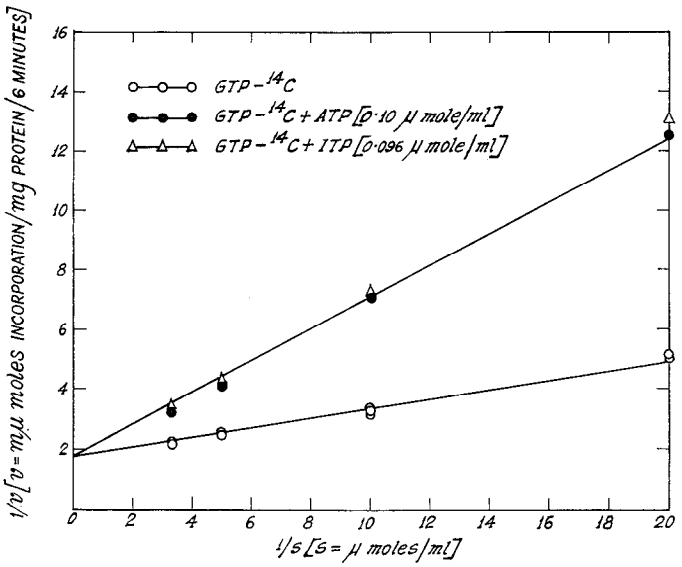

FIG. 5. Double reciprocal plot of reaction rate (millimicromoles of incorporation per mg of protein per 6 min at 37°) against substrate concentration (micromoles per ml of reaction mixture). The effect of ATP and ITP on GMP incorporation is also shown. Incubation conditions and assay procedure were as described under "Methods."

TABLE III

Effect of pyrophosphate, orthophosphate, GDP, and GMP on incorporation of GMP from GTP

GTP-8-^{14}C, 0.4 μmole, 0.2 μmole of GM^{32}P (3 × 10^6 cpm per μmole), and 1.0 μmole each of nonradioactive GMP and GDP were used per ml of reaction mixture. Other reaction conditions and assay procedure are mentioned under "Methods."

Substrate and addition	Incorporation
	mμmole/mg protein
GTP-8-^{14}C	
None*	0.30
Pyrophosphate,* 20 μmoles	0.07
None	0.68
Orthophosphate, 20 μmoles	0.66
GDP	0.66
GMP	0.67
GM^{32}P	
None	<0.05

* Succinate buffer, pH 6.3, was used in this case.

TABLE IV

Effect of different polynucleotides on GMP incorporation

The enzyme was heated to 80° for 5 min and Extract I was obtained as the supernatant of the heated enzyme on centrifugation. Extract II was prepared by phenol treatment of the enzyme which was shaken with 90% phenol (equal volume) for 20 min. After centrifugation, the aqueous layer was dialyzed against several changes of distilled water in the cold before use. Other ingredients as mentioned under "Methods" have been used.

Polynucleotide added*	Incorporation
	mμmole/mg protein
None	0.70
Spinach leaf RNA	0.56
Spinach leaf DNA	0.52
Calf thymus DNA	0.50
Poly A	0.42
Poly C	0.29
Poly U	0.71
Extract I	0.82
Extract II	0.86

* The amount added in each case was 25 μg per ml.

Effect of Nucleic Acids—In order to elucidate the primer requirement for GMP incorporation, a number of polynucleotides including naturally occurring RNA and DNA from different sources as well as enzymatically synthesized homopolymers of AMP, UMP, and CMP have been used. The pertinent data presented in Table IV show that except for poly U and the extracts from the enzyme preparation itself, all of the other polynucleotides including DNA are inhibitory to the reaction when used at a concentration of 25 μg per ml. There is a slight increase of activity in extracts containing RNA obtained from the enzyme preparation by heat precipitation of protein or phenol treatment. A higher concentration (50 μg per ml) of these extracts does not further stimulate activity.

Fig. 6 represents the inhibition of GMP incorporation by poly A and poly C at different concentrations.

The inhibition, especially that by poly C, is unexpected. However, a partial reversal of inhibition by polynucleotides can be obtained by using an increased concentration of Mn^{++}. Thus, 63% inhibition by 50 μg per ml of poly C in the presence of 5 μmoles per ml of Mn^{++} can be reduced to 40% when 15 μmoles per ml of Mn^{++} are used. The fact that complete reversal of inhibition is not observed with higher concentrations of Mn^{++} and that the extent of inhibition by different polynucleotides is very dissimilar (poly U not inhibiting at all when used in a concentration of 25 μg per ml) indicate that factors other than cation binding may also be involved.

Effect of RNase and DNase—To demonstrate the effect of pancreatic RNase and DNase, the enzyme preparation was preincubated for 10 min with RNase or DNase followed by incubation with GTP-8-^{14}C. The results, presented in Table V, indicate that there is substantial reduction in incorporation when the enzyme is preincubated with RNase but not with DNase. This may be due to the fact that the primer RNA is susceptible to pancreatic RNase. Another observation of interest has been the considerable loss of activity due to preincubation alone without any added RNase (control). It has been established that this loss of activity is not due to the simultaneous presence of Mg^{++} and Mn^{++} in the incubation mixture.

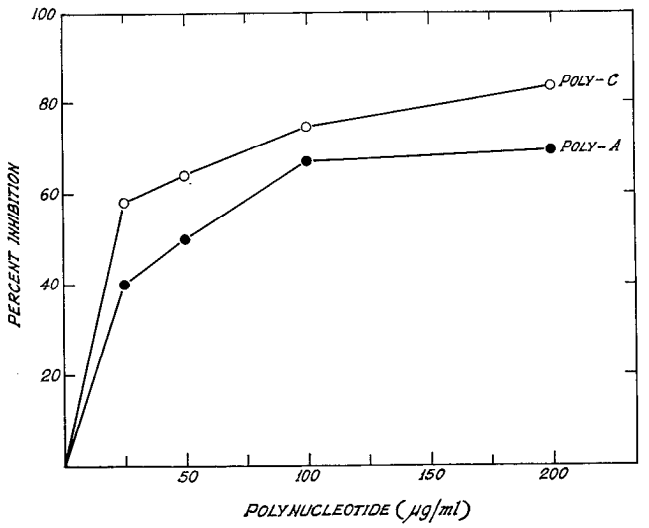

FIG. 6. Inhibition of GMP incorporation by different concentrations of poly A and poly C at a constant concentration of $MnCl_2$ (5 μmoles per ml) and of GTP-8-^{14}C (0.4 μmole per ml). Other conditions and assay procedure were as described under "Methods." By 100% incorporation is meant the incorporation of 0.70 mμmole of GMP-8-^{14}C in control under standard conditions without any added polynucleotide (Table IV).

TABLE V

Effect of prior incubation with pancreatic RNase and DNase

The preincubation mixture contained, in a final volume of 0.5 ml, Tris buffer, pH 7.20, 25 μmoles, and the enzyme, 1.5 mg of protein ("Control"). In a second tube, 5 μg of pancreatic RNase and in a third tube, 5 μg of DNase plus 3 μmoles of $MgCl_2$ were present in addition to the buffer and enzyme. After 10 min at 37°, 100 μmoles of Tris buffer, pH 8.50, 4 μmoles of $MnCl_2$, and 0.4 μmole of GTP-8^{14}-C were added to all of the samples and 3 μmoles of $MgCl_2$ were added to the control and RNase-treated samples. The final reaction volume was made to 1 ml and incubation was allowed to proceed for 15 min at 37°. After incubation, the reaction was stopped and assayed as indicated under "Methods."

Conditions	Incorporation
	mμmole/mg protein
Control	0.49
RNase	0.08
DNase	0.50

The loss of activity due to preincubation was further investigated to determine the effect of various added polynucleotides on the preincubated enzyme. In these experiments, the enzyme was preincubated at 37° for 20 min with all of the ingredients except the substrate and the polynucleotide. After preincubation, 0.4 μmole of GTP-8-^{14}C was added, followed by incubation for 15 min at 37°. The results, presented in Table VI, indicate that while RNA as well as DNA are largely ineffective, restoration of activity can be accomplished with the polynucleotides extracted from the enzyme preparation itself, irrespective of the manner in which the extraction is made. That the RNA present in the extract is responsible for this restoration is inferred from the fact that its brief exposure to pancreatic RNase results in complete loss of restoration capacity. The lack of effect of several other RNA preparations indicates that the primer is somewhat specific. Thus, even unfractionated RNA from spinach leaf is without any restoring capacity.

The data presented in Tables V and VI suggest the participation of a polynucleotide primer. The fact that DNA, either native or denatured, cannot restore the loss of activity due to preincubation as well as the fact that DNase fails to inhibit GMP incorporation indicates that DNA is not involved in this reaction. This has been further supported by studies with actinomycin D which is known to inhibit DNA primed RNA synthesis by complexing with the dGMP residues of the DNA molecule (33). Actinomycin D, up to a concentration of 50 μg per ml, does not inhibit the incorporation of GMP.

Analysis of Product—The radioactive product formed by the enzyme with GTP-8-^{14}C was hydrolyzed with alkali and was analyzed as described under "Methods." The data given in Table VII indicate that incorporation is largely internal. No activity has been recorded in the guanosine 2′(3′)5′-diphosphate fraction, and almost all of the radioactivity can be accounted for in the guanosine and guanosine 2′(3′)-phosphate fractions.

The radioactive product, isolated with phenol (30), has also

TABLE VI

Effect of polynucleotides on activity of preincubated enzyme

For each polynucleotide, 25 to 30 μg were used. Preparation of Extract I and Extract II has been described in the legend of Table IV. Extract III was prepared by homogenizing the perchloric acid (1 M)-precipitated enzyme with 0.1 M Tris, pH 7.6, followed by centrifugation in the cold. The 280:260 absorbance ratios of the extracts were: Extract I, 0.80; Extract II, 0.68; Extract III, 0.72. Standard incubation conditions and assay procedure have been followed.

Conditions	Incorporation
	mμmole/mg protein
Control (without preincubation)	0.65
Preincubated for 20 min	0.39
+Spinach leaf RNA	0.30
+Chloroplast DNA	0.29
+Denatured DNA (thymus)*	0.27
+Extract I	0.60
+Extract II	0.62
+Extract III	0.62
+Poly U	0.42
+Poly C	0.30
+Poly A	0.33

* DNA, dissolved in 0.05 M Tris, pH 7.5, containing 0.01 M NaCl, was denatured by heating at 100° for 10 min followed by rapid chilling in an ice bath.

TABLE VII

Terminal and internal incorporation

The radioactive product, containing about 4500 cpm and 3000 cpm, has been analyzed by Method I and Method II, respectively. Details of experimental procedure are indicated under "Methods."

Products of hydrolysis	Total radioactivity recovered by		Radioactivity recovered (average)
	Method I	Method II	
	cpm		%
Guanosine	215	120	4–5
Gp	4100	2570	95
pGp		20	<1

been tested for its stability under various conditions, especially for its susceptibility to pancreatic RNase, DNase, RNase T_1, and alkali. A portion of the product (2000 to 3000 cpm) was incubated for 2 hours at 30° alone (control) or with 100 μg per ml of pancreatic RNase. After incubation, the samples were dialyzed against either distilled water or 0.05 M Tris, pH 7.2, for the desired period. A similar portion of the product was treated with RNase T_1 (100 μg per ml) under appropriate conditions of pH (7.5) and EDTA concentration (2 μmoles per ml). Similarly, DNase (100 μg per ml) treatment was carried out in the presence of 5 μmoles per ml of $MgCl_2$ at pH 7.2. The results depicted in Table VIII show the resistance of the reaction product to pancreatic RNase and, as expected, to DNase. Susceptibility to RNase T_1 on the other hand suggests the formation of guanylate and guanylate linkages since RNase T_1 is known to specifically attack the phosphodiester bonds involving guanylic acid residues which are resistant to pancreatic RNase. Even prolonged treatment with RNase T_1, however, does not liberate all of the radioactivity, about 20% of it being retained. This may indicate the existence of secondary hydrogen bonding in the polynucleotide molecule, which would confer resistance to enzymatic hydrolysis on the polynucleotide concerned. Complete liberation of all of the radioactivity on alkaline hydrolysis further confirms that the product is a ribopolynucleotide.

Nearest Neighbor Frequency—Experiments to determine the nearest neighbor frequency were done after incubation with $GT^{32}P$ alone as well as with $GT^{32}P$ plus ATP (1:1). The results (Table IX) indicate that while most of the GMP units occur next to GMP (at least 90%), the most favored nonguanylate residue with which GMP seems to be associated is UMP. The presence of ATP in the incubation mixture does not appreciably change these relations, indicating thereby that the formation of the random copolymer poly AG does not occur.

TABLE VIII

Stability of product

Isolation of the radioactive product, incubation, and dialysis were done as described in the accompanying text.

Incubation with	Time	Dialysis* against	Time	Radioactivity lost
	hrs		*hrs*	*% original*
None		Distilled water in cold (0–4°)	20	2
At 30°	2	Distilled water at 30°	2	5
Pancreatic RNase (100 μg per ml) at 30° with 50 μmoles per ml of Tris, pH 7.2	2	50 μmoles per ml of Tris, pH 7.2, at 30°	2	6
DNase (100 μg per ml) at 30° with 50 μmoles per ml of Tris, pH 7.2, and 5 μmoles per ml of $MgCl_2$		50 μmoles per ml of Tris, pH 7.2, plus 5 μmoles per ml of $MgCl_2$ at 30°	2	5
RNase T_1 (100 μg per ml) at 30° with 50 μmoles per ml of Tris, pH 7.5, and 2 μmoles per ml of EDTA	2 2	50 μmoles per ml of Tris, pH 7.5, plus 2 μmoles per ml of EDTA at 30°	2 20	75 80
500 μmoles per ml of KOH at 37°	18	Distilled water at 30°	6	100

* In all of the cases, dialysis was carried out against 1 liter of either distilled water or the solutions as indicated.

TABLE IX

Nearest neighbor relationships after incorporation of $GM^{32}P$

The 2′(3′)-nucleotides were isolated from the alkaline hydrolysate of the reaction product either with Dowex 1-formate (31) or by paper electrophoresis with Whatman No. 3MM paper. Electrophoresis was carried out in 0.05 M ammonium formate buffer, pH 3.8, under potential gradient of 13 volts per cm for 4 hours. The nucleotides were assayed for radioactivity, and the dinucleotide frequency was determined (30).

Substrate and dinucleotide sequences	Relative frequency
$GT^{32}P$	
ApG	0.01
CpG	<0.01
UpG	0.05
GpG	0.94
$GT^{32}P$ + ATP (1:1)	
ApG	0.03
CpG	<0.01
UpG	0.05
GpG	0.92

DISCUSSION

Chloroplasts are self-duplicating organelles that have been shown to contain DNA (28, 34, 35) and an independent RNA-synthesizing machinery (36–38). Extracts of chloroplasts from spinach leaf have been found to catalyze the synthesis of a variety of homopolymers (23). The preparation and some of the distinguishing properties of a fraction obtained from grana which is responsible for the predominantly nonterminal incorporation of GMP into polynucleotide material have been described in this report.

Incorporation of GMP from GTP is inhibited by ATP and also by ITP (Table I and Fig. 5). While UTP and CTP do not inhibit the incorporation of GMP, poly U at higher concentrations and poly C at a comparatively low concentration inhibit GMP incorporation, perhaps indicating that the inhibition is due mainly to polynucleotides. This may also be applicable in the case of ATP and ITP as the same fraction can catalyze the incorporation of AMP and IMP. Besides these homopolymers, a number of RNA and DNA preparations have also been found to inhibit GMP incorporation (Table IV). The mechanism underlying the inhibition by polynucleotides in the present case is not known with certainty. The partial reversal of inhibition by poly C in the presence of increased Mn^{++} may be due to an effect on the solubility or aggregation of poly C rendering it a less efficient inhibitor. That the polymerization reactions of polynucleotide phosphorylase are inhibited by complementary homopolymers has been reported by Heppel (39). The inhibition of the activity of purified RNA polymerase by polynucleotides has also been demonstrated by Krakow and Ochoa (19), Fox *et al.* (20), and Fox and Weiss (40). The enzyme system described here is, however, distinct from RNA polymerase in that it does not carry out the production of a ribopolynucleotide complementary in nucleotide composition to the primer.

The reaction is dependent on the presence of a divalent cation.

The incorporation in its absence may represent nonenzymatic binding of GTP. However, the partial promotion by Cu^{++} is peculiar to this system since no other polymerase is known to be stimulated by Cu^{++}.

The enzyme preparation contains 35 to 40 μg of RNA per mg of protein. After preincubation for 20 min there occurs about 40% loss of activity which is fully restored by RNA isolated from the enzyme system itself. Neither RNAs obtained from different sources nor DNA, even preparations isolated from chloroplasts, can replace the RNA extracted from the enzyme preparation in this respect.

The absence of radioactivity in guanosine 2′(3′)5′-diphosphate in the alkaline hydrolysate of the reaction product or in any slower moving spot (which might have arisen from a 5′-triphosphate-ended poly G chain) suggests that free poly G is not synthesized. It is concluded from the nearest neighbor frequency data that the primer is perhaps a ribopolynucleotide with a UMP residue at the acceptor end.

The number of GMP residues added at the end of the primer is not more than 20 as calculated from the data of Table VII, assuming linear extension of the chain. That longer chains are not synthesized may be due to the well known tendency of guanine oligonucleotides to assume secondary structures (41). The observed resistance of a part of the reaction product to hydrolysis by RNase T_1 supports this contention. It has been demonstrated by Fresco and Su (22) that the primer RNA may have some influence on this secondary structure formation until a minimum chain length has been reached.

The nearest neighbor frequency data also rule out the possibility of any copolymer formation and indicate the independent incorporation of GMP and AMP into separate chains. As has already been pointed out, it is not known whether the same enzyme is responsible for the incorporation of GMP, AMP, and also of IMP. It can, however, be speculated that if the same enzyme is involved, specificity may lie in the primer polynucleotide. Further purification of the enzyme system is necessary before any definite conclusion can be drawn. This has been attempted, so far without success, the failure being partly attributable to the very poor solubility of the chloroplast proteins once sedimented. However, it has been observed[2] that some of the characteristics of the reaction catalyzed by the same preparation with ATP as substrate are different from those reported here with GTP. The significance of these observations is at present being studied in this laboratory.

SUMMARY

An enzyme system extracted from the grana fraction of spinach chloroplasts has been shown to catalyze the incorporation of guanosine phosphate from guanosine triphosphate into polynucleotide material. The same fraction can also carry out the incorporation of adenosine and inosine phosphates and the respective nucleoside triphosphates inhibit the incorporation of guanosine phosphate.

Incorporation of GMP requires the presence of a preformed ribonucleic acid primer which is somewhat specific, all of the other polynucleotides tested being inhibitory. The primer is presumably a ribopolynucleotide with a UMP residue at the acceptor end where GMP units are added, the number of GMP residues incorporated per chain being not more than 20. Besides the primer, the enzyme requires a suitable cation, preferably Mn^{++}, for optimal activity.

GMP incorporation is susceptible to ribonuclease but not to deoxyribonuclease. DNA has been found to be inhibitory when added to either preincubated or non-preincubated enzyme. Actinomycin D (50 μg per ml) does not inhibit the reaction.

The incorporated GMP is acid-precipitable, nondialyzable, and resistant to pancreatic RNase and DNase, but it is susceptible to RNase T_1 and is completely alkali labile. The nearest neighbor frequency analysis shows that more than 90% of the GMP units occur next to GMP. The formation of the random copolymer of adenylic and guanylic acids does not take place in the simultaneous presence of $GT^{32}P$ and ATP in the reaction mixture; instead, separate chains are synthesized.

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[2] S. Biswas and B. B. Biswas, in preparation.

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