The Absence of Phosphorylated Chain Ends in Tobacco Mosaic Virus Ribonucleic Acid*

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Alkaline degradation of P^{32}-labeled tobacco mosaic virus ribonucleic acid (TMV-RNA) yields no nucleoside 2′(3′),5′-diphosphates, which signifies the absence of 5′-phosphorylated chain ends. Degradation by snake venom diesterase yields under optimal conditions less than one mole of nucleoside 2′(3′),5′-diphosphates, which signifies the absence of such 2′(3′)-phosphorylated chain ends. The variable amounts of nucleoside 2′(3′),5′-diphosphates and of the corresponding nucleoside 2′3′(cyclic),5′-diphosphates found are attributed to chain breakage due to contaminating nucleases. Phosphomonoesterase releases phosphate from typical RNA preparations, but almost none from two types of specially purified RNA preparations. It appears that small amounts of noninfective material rich in terminal phosphate can be largely removed either by incubation of the virus with chelating agents or by fractionation of the RNA on a sucrose gradient. The data presented indicate that neither the terminal 5′-position nor the terminal 2′(3′)-position of the RNA chain carries a phosphate group.

The ability of viral RNA to carry and transmit genetic information is now generally attributed to the sequential arrangement of the bases along the polynucleotide chain. Methods for the determination of nucleotide sequences are as yet quite inadequate, and the chain length of the smallest viral nucleic acid known (about 5000 nucleotides) appears to preclude any such analysis.

However, protein structure analysis appeared a similarly hopeless task 20 years ago, and now it is a firmly established field of endeavor. It therefore seems justified to attack the problem of analyzing RNA by focusing attention on attainable first objectives and hoping that a fortuitous combination of developments may gradually reduce the seeming limitlessness of the assignment. Protein structure analysis received its main impetus from the development of methods for end-group analysis and stepwise degradation. These methods have also supplied important information concerning the chemical homogeneity of protein preparations. A similar line of attack on the RNA problem, starting from the chain ends, thus seems reasonable. In an unbranched polynucleotide of the molecular weight of TMV-RNA (2.1×10^6, 6500 nucleotides) only 0.03% of the nucleotides would be expected to be in terminal position. Thus, 21 mg of viral RNA, representing a major expenditure of precious material (about 0.5 g of the tobacco mosaic virus), would yield only 0.01 μmole of an end group (*e.g.*, 1 μg of a pyrimidine). This seemingly formidable analytical difficulty is overcome by the use of RNA isolated from virus grown in the presence of, and thus heavily labeled with, radioactive isotopes. With virus containing enough P^{32} or C^{14} (about 10,000 cpm/μg RNA), fractional amounts of one terminal group can be detected, measured, and characterized in experiments conducted with only 0.1-1 mg of RNA.

The end-group methods employed in this study are based on well-established principles. As illustrated on Figure 1, alkaline degradation of RNA yields a mixture of 2′ and 3′-nucleotides, one nucleoside diphosphate from the 3′-linked end of the molecule if that was phosphorylated, and one nucleoside from the 5′-linked end if that was unphosphorylated. On the other hand, degradation by snake venom diesterase yields 5′-nucleotides, one potential diphosphate from the 5′-linked end, and one potential nucleoside from the 3′-linked end. The search for the terminal nucleosides, which requires C^{14}-labeled virus, represents a separate study (Sugiyama and Fraenkel-Conrat, 1961). The absence of detectable nucleoside 2′(3′),5′-diphosphates from alkaline digests of TMV-RNA has previously been reported (Reddi and Knight, 1957; Matthews and Smith, 1957). Only now, however, has this been established with a sufficiently sensitive method to permit the conclusion that the 5′-position on the 3′-linked terminal nucleotide of TMV-RNA

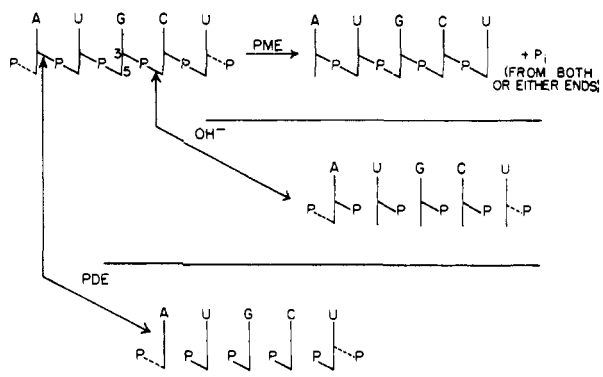

FIG. 1.—Principal methods of end-group analysis of RNA. *Top*, the releasing of terminal phosphates (if any) as inorganic phosphate (P_i) by phosphomonoesterases (PME). *Below*, the attack by 0.1–1 N NaOH (OH^-), which breaks the 5′-phosphate linkage and produces 3′-nucleotides; and by snake venom phosphodiesterase (PDE), which breaks the 3′-linkage and yields 5′-nucleotides. Both agents may yield 0, 1, or 2 characteristic products from end groups (a nucleoside and/or a nucleoside diphosphate), depending on their state of phosphorylation.

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of molecular weight of 2 million is unphosphorylated.

The degradation of nucleic acid by snake venom diesterase often gives somewhat equivocal results, since it is necessary to incubate the RNA under conditions in which traces of ribonuclease-like enzymes, or hydroxyl ions, can create cyclic 2′,3′-phosphate ends which then appear as doubly phosphorylated nucleosides after complete degradation of the RNA. This danger has often been recognized and never completely abolished (Crestfield and Allen, 1956; Heppel and Rabinowitz, 1958). This probably accounts for the fact that low though somewhat variable amounts of the nucleoside 2′(3′),-5′-diphosphates were found by us. Recent improvements in the preparation of the RNA (Fraenkel-Conrat *et al.*, 1961) have further lowered the nucleoside 2′(3′),5′-diphosphate content of diesterase digests. Under the most favorable conditions of digestion and chromatographic separation, the total amount of the two types of products, the nucleoside 2′(3′),5′-diphosphates and the nucleoside 2′3′(cyclic),5′-diphosphates, duly corrected for losses, has been less than one residue per mole of RNA in several experiments. It thus seems probable that the frequent finding of greater amounts of these substances represents the remainder of what has been the problem throughout, namely, the presence of artifact ends caused by chain breakage, be it enzyme or alkali catalyzed, in the course of incubation. This belief is supported by the observation that the nucleoside 2′3′(cyclic),5′-diphosphates were almost always present and in the case of cytidine 2′3′(cyclic),5′-diphosphate were predominant in amount over the open form. The fact that both pyrimidine diphosphates were generally present in approximately equal amounts, even when their total amount was quite low, supports the belief that they were not derived from a true end group. Much less material was always found in the area of the purine 2′(3′),5′-diphosphates than in that of the pyrimidine derivative.

Since the existence of terminal 5′- and 3′-phosphate groups in TMV-RNA was seemingly ruled out by application of the methods described, a reinvestigation of the action of phosphomonoesterases appeared indicated. In a previous paper it was reported that prostatic monoesterase released no more than one, but seemingly one, residue of inorganic phosphate per mole of TMV-RNA (Gordon *et al.*, 1960). Since then our RNA preparations had been improved, in particular in regard to freedom from nucleases. Further, a highly purified bacterial esterase had been described by Garen and Levinthal (1960). A sample of this material was therefore obtained, and its action on TMV-RNA was studied. It appeared that RNA (as customarily prepared by our older methods) was attacked by the bacterial monoesterase, releasing about 2–3 gram equivalents of inorganic phosphate per 2.1×10^6 g RNA over a wide range of enzyme concentration and time. This release, slightly higher than that obtained with the prostatic enzyme, was not accompanied by a loss of infectivity of the RNA if the amount of enzyme and time of incubation were not in excess of that required for maximum release of phosphate. This and other indications demonstrated the absence of any detectable diesterase or nuclease in the bacterial esterase preparation at the concentration levels used. However, the origin of the relatively high amount of inorganic phosphate released was enigmatic. On the basis of recurring concern that viral RNA might contain some fragments or shorter oligonucleotides rich in end groups, attempts to fractionate the material were again initiated. In contrast to earlier attempts with salt fractionation, which were not readily reproducible (Gordon *et al.*, 1960), centrifugation in a sucrose gradient supplied a consistently successful method of fractionating those RNA preparations which upon monoesterase treatment yielded appreciable amounts of inorganic phosphate. The RNA was found only in the top one-half of the gradient tube, and here the distribution of RNA was approximately gaussian. The bulk of this material was usually the most highly infective and released very little phosphate upon phosphomonoesterase treatment. In contrast, the trailing material, usually of lower infectivity, was enriched in esterase-susceptible phosphate groups.

Concurrent studies by Whitfeld (1961) showed that incubation of P^{32}-containing virus with citrate buffer (pH 6) caused the release of about 1–2% of the P^{32} into the nonsedimentable supernatant solution. When virus preparations were pretreated in this way with citrate buffer (or similarly with versene buffer) and then treated by the recently developed bentonite-phenol procedure (Fraenkel-Conrat *et al.*, 1961), the RNA obtained proved as low in monophosphatase-susceptible sites as did the fractions obtained by centrifugation with a sucrose gradient. It thus appears probable that the infective, unfragmented viral polynucleotide chain lacks monoesterified phosphate groups. The data obtained with monoesterase do not exclude the possibility of the existence of a 2′,3′-cyclic terminal phosphate group, but this possibility does seem to be excluded by some of the results of the diesterase and snake venom experiments.

These three lines of research—degradation with alkali, degradation with diesterase, and treatment with phosphomonoesterase—thus lead to the conclusion that both terminal residues of TMV-RNA are unphosphorylated. With P^{32} as a label this conclusion is of necessity based on negative evidence. The technical problem of excluding the presence of a single terminal phosphate among 6500 internal ones is compounded by the existence in viral RNA preparations of small amounts of fragments and, possibly, contaminants which bear terminal groups. Thus not the highest, but the lowest, reproducible values obtained are the most significant, if error and losses can be excluded or corrected in unambiguous manner. While such low reproducible values after appropriate correction have been achieved in this study, it is nevertheless comforting that current experiments with C^{14}-labeled virus are supplying support to the conclusion that both terminal residues of TMV-RNA are unphosphorylated by making possible the positive identification of the terminal nucleosides (Sugiyama and Fraenkel-Conrat, 1961).

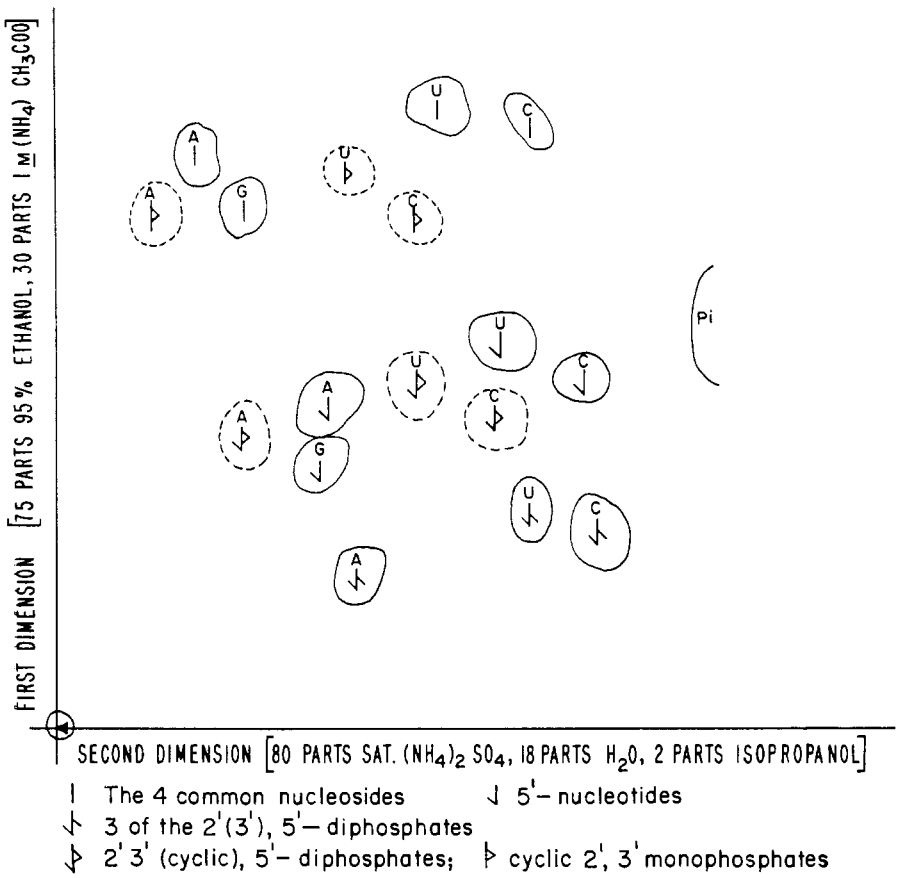

FIG. 2.—Chromatographic separation of split products of RNA (Felix *et al.*, 1960). The spot labeled P_i (inorganic phosphate) delineates the second front. The first is further than shown, the nucleosides having approximate R_F values of 0.7 in the first dimension. 3'(2')-Nucleotides are not shown. These isomers usually separate well from each other, though not necessarily from the 5'-nucleotides.

It must be noted that the absence of terminal phosphates is a property of the RNA as isolated, but that prior dephosphorylation by a plant monoesterase has not been ruled out. However, the methods involved in phenol degradation of the virus and isolation of the RNA make such a dephosphorylation appear very unlikely. But direct alkaline degradation of the virus may be feasible, and it is planned to test for the presence of a 5'-phosphorylated terminal nucleotide in the virus in this manner. In view of the now established mode of synthesis of RNA from 5'-triphosphates, it seems somewhat surprising that the 5'-terminal residue to TMV-RNA bears no phosphate.

METHODS AND MATERIALS

P^{32}-TMV was prepared as previously described (Gordon *et al.*, 1960). The RNA was prepared from it by the phenol procedure, and during the past year almost always in the presence of bentonite (Fraenkel-Conrat *et al.*, 1961).

A sample of purified *Escherichia coli* phosphatase (Garen and Levinthal, 1960) was kindly supplied by Dr. C. Levinthal. Several preparations of purified snake venom (*Bothrop atrox*) phosphodiesterase (Felix *et al.*, 1960; Williams *et al.*, 1961) were kindly sent us by Dr. M. Laskowski. They were mailed and stored frozen.

Digestion with bacterial monoesterase was performed at 36° for 30 minutes in about 0.5×10^{-2} M pH 7.3 Tris or ammonium acetate containing about 10^{-3} M Mg^{++}; the diesterase was usually employed in borate buffers (pH 8.3–9.5) containing 10^{-3} M Mg^{++}. Crude snake venom, a 1% dispersion of commercial *Crotalus adamanteus* venom, centrifuged and stored frozen, was employed under the same conditions. Primary fractionations were frequently performed by paper electrophoresis (0.05 M phosphate, pH 7.4), as previously described (Sugiyama and Fraenkel-Conrat, 1961; Gordon *et al.*, 1960). Alkaline digests were often neutralized with phosphate and directly applied to the paper, if the total volume did not exceed 0.3 ml.

For chromatographic separations of all four nucleosides and nucleotides, as well as of the three nucleoside diphosphates and cyclic nucleoside diphosphates available to us, the two-dimensional system of Felix *et al.* (1960) proved very advantageous (see Fig. 2). The only difficulty with this technique was that the extent of movement in the first dimension was quite variable. Eluates from such chromatograms were so rich in ammonium sulfate that they had to be desalted before their radioactivity was counted. Desalting was also necessary prior to application of some digests to

the paper. Charcoal adsorption of the purine and pyrimidine compounds was utilized for this purpose. The entire aqueous eluate or sample was adjusted to pH 2 with N HCl in the presence of 5–10 mg of acid washed Norite A. After a few minutes the charcoal mixture was centrifuged in a clinical centrifuge, the salt-containing supernatant solution poured off, and the charcoal resuspended in H_2O and again centrifuged to remove remaining acid and salt. Nucleotides and nucleosides were then eluted from the charcoal by addition of 2 ml 2% NH_4OH in 50% ethanol-H_2O. More than one elution was usually necessary for maximum recovery.

Samples of cytidine 2′(3′),5′-diphosphate and uridine 2′(3′),5′-diphosphate were kindly supplied by Dr. C. A. Dekker, and a sample of adenosine 3′,5′-diphosphate was kindly supplied by Dr. J. Baddiley. (These compounds will be symbolized "pCp," "pUp," and pAp.[1]) Another sample of adenosine 3′,5′-diphosphate was prepared from coenzyme A by the method of Wang *et al.* (1959). Unfortunately the coenzyme and adenosine 3′,5′-diphosphate showed the same electrophoretic mobility and the same R_F in all chromatographic solvents tested with the possible exception of a small difference in ethanol–ammonium acetate (Felix *et al.*, 1960). However, the ratio of phosphorus found to UV absorption indicated that the product was the expected pAp.

Cyclization of the three diphosphates was performed by a modification of the methods used by Dekker and Khorana (1954) and by Shugar and Wierzchowski (1958) for the preparation of 2′3′-cyclic phosphates. Eight mg of nucleoside 2′(3′),5′-diphosphate was dissolved in 0.2 ml H_2O with addition of HCl to pH 5–6. Five-tenths ml *N,N*-dimethylformamide was added and the solution again adjusted to an apparent pH of 5–6 with HCl. To the clear solution was added 500 mg *N,N′*-dicyclohexylcarbodiimide in 0.5 ml *N,N*-dimethylformamide. *N,N′*-Dicyclohexylurea began to crystallize after a few minutes at room temperature. The solution was kept at room temperature 16 hours, diluted with 1 ml H_2O, and the copious precipitate centrifuged off. The clear supernatant was then ether extracted several times. The cyclization of cytidine 2′(3′),5′-diphosphate, uridine 2′(3′),5′-diphosphate, and adenosine 3′,5′-diphosphate proceeded in near-quantitative yield as shown by chromatography in ethanol-ammonium acetate (75 ml 95% ethanol–30 ml 1 M pH 7.5 ammonium acetate on Whatman 3 MM paper 20 hours). The cyclic diphosphates showed higher R_F values than the open forms and lesser R_F values than the corresponding mononucleotides. They were isolated by chromatography with the above solvent and subsequent elution with H_2O. They will be symbolized as pC > p, pU > p, and pA > p.

Identification of these compounds as the nucleoside 2′3′(cyclic),5′-diphosphates was attempted within the limits imposed by the small quantities available. After phosphomonoesterase treatment, which removed phosphate, the compounds on two-dimensional chromatography were identical with the corresponding cyclic 2′,3′-monophosphates. On alkaline hydrolysis the phosphomonoesterase-treated compounds corresponded to a mixture of 2′- and 3′-mononucleotides. Before phosphomonoesterase treatment, alkaline hydrolysis yielded a material chromatographically identical to the nucleoside 2′(3′),5′-diphosphate markers.

The possibility that the synthesized compound contained a pyrophosphate link is ruled out by the fact that such a link would not be broken by phosphomonoesterase and would therefore not be dephosphorylated. The behavior of the synthesized compounds appears to be consistent with the proposed 2′3′(cyclic),5′-diphosphate structure and not a nucleoside 3′5′-cyclic pyrophosphate.

Results

Degradation of P^{32}-Labeled TMV-RNA with Alkali.—Many experiments were performed in which 0.18–1.0 mg of TMV-RNA containing sufficient P^{32} to give at least 10^6 cpm was treated with KOH to a final concentration of 0.03 to 1.0 N, equivalent to 3–1000 times the phosphorus present. In most experiments about 0.1 μmole of at least one and usually two of the nucleoside diphosphates (pAp, "pUp," "pCp") was added prior to incubation of the alkaline digest (usually 18–24 hours at 25° or 36°). The digests were then worked up and fractionated in various ways: (1) They were brought to about pH 7 with $HClO_4$, the $KClO_4$ precipitate was centrifuged off, and the supernatant solution was concentrated and subjected to electrophoresis (pH 7.4 phosphate). (2) They were neutralized with 0.1 M phosphate buffer and directly subjected to electrophoresis without removal of any insoluble precipitate. Radioautographs of the electropherograms showed that the P^{32} was predominantly in the area of the mononucleotides. No P^{32} spots coincided with the location of the added carrier diphosphate markers detected under UV light, although amounts corresponding to one diphosphate per mole should have been clearly discernible. The marker spots, as well as adjacent areas, were cut into strips and eluted, the recovery of the markers was established spectrophotometrically, and the radioactivity in the eluates was determined. Frequently the radioactivity in the paper strips was determined directly, without elution, since P^{32} counts are almost identical whether counted as a film or dried on paper. Proceeding from the very high radioactivity in the nucleotide area there was a more or less steep decrease in cpm per cm strip (~4 cm^2). Thus the observed low counts cannot be attributed to diphosphates, whereas the presence of a trace of inorganic phosphate (about 0.1 mole/mole RNA) could be unequivocally detected on several such electropherograms. The total radioactivity in any one diphosphate marker area, corrected for the recovery of the marker (50–90%), usually amounted to no more than 0.01% of the phosphorus, while 0.03% of the phosphorus would be present in one terminal diphosphate per mole of RNA. Even though no guanosine 2′(3′),5′-diphosphate was available as a

[1] Normally the abbreviation pCp designates only the nucleoside 3′,5′-diphosphate. The use of quotation marks signifies that these compounds are either mixtures or possible mixtures of the 2′,5′- and the 3′,5′-diphosphates.

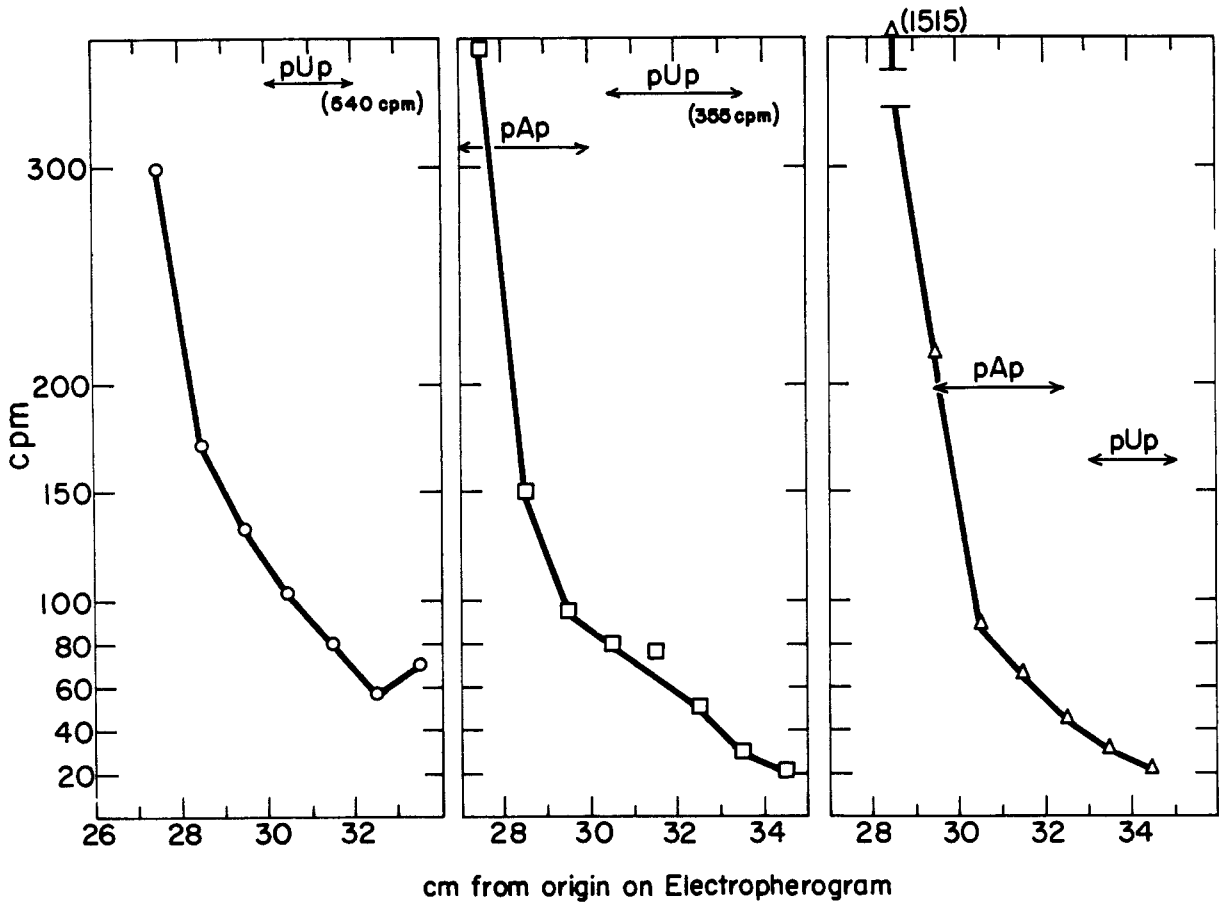

FIG. 3.—Absence of nucleoside 2′(3′),5′-diphosphates from NaOH digests of TMV-RNA. The data of three separate experiments are given. Each graph represents the pattern of radioactivity (cpm) per cm strip from paper electrophoretic separation of the digests. In each the location of the unlabeled nucleoside 2′(3′),5′-diphosphates added as markers is indicated ("pUp," pAp). The expected radioactivity for one terminal diphosphate (0.03% of the total cpm used in the given experiment, divided by the recovery of absorbancy of the marker) is indicated by the vertical position, or cpm number of the markers. The bulk of the P^{32} (10^6 cpm) is in the nucleotide area just preceding the nucleoside 2′(3′),5′-diphosphate markers, and its trail is incorporated in the first strips counted.

marker when these experiments were performed, the presence of this substance would surely have been revealed by the techniques employed.[2] A few typical experiments are summarized in Figure 3.

Occasionally, the neutralized digests were subjected to two-dimensional chromatography. Radioautographs of these chromatograms showed the expected very dark 2′- and 3′-nucleotide areas, as well as a small amount of streaking across the nucleoside 2′(3′),5′-diphosphate areas. The diphosphate marker spots were not labeled, but on account of the streaking from the highly labeled nucleotides this technique was found inferior to electrophoresis for the purpose of quantitatively establishing the presence or absence of traces of nucleoside 2′(3′),5′-diphosphates in a mixture of nucleotides.

Degradation of P^{32}-Labeled TMV-RNA with Snake Venom Enzymes.—When 0.2–8 units of highly purified snake venom diesterase (Felix *et al.*, 1960; Williams *et al.*, 1961) were allowed to act on 0.2–1 mg TMV-RNA ($\sim 10^6$ cpm) at pH 8.5–9.5 in the presence of Mg^{++} in a pH-stat or in borate buffers of various molarity, digestion to 5′-nucleotides approached completion in 1–2 hours at 36°. While the chromatograms of such digests showed only very minor amounts of scattered radioactivity, apparently a consequence of incomplete digestion, as well as some trailing from the heavily labeled 5′-nucleotide spots, this material nevertheless interfered with the quantification of traces of nucleoside 2′-(3′),5′- and nucleoside 2′3′(cyclic),5′-diphosphates. This was circumvented by either of two procedures. One consisted in adding crude snake venom (*Crot. adam.*) to the digests resulting from the action of the purified diesterase. The 5′-nucleotidase in this venom dephosphorylated the nucleotides and transformed to inorganic P^{32}-phosphate all but the nucleotidase-resistant nucleoside 2′(3′),5′- and nucleoside 2′3′(cyclic),5′-diphosphates derived from chain ends. Charcoal adsorption and elution separated the purine and pyrimidine derivatives from the bulk of the now inorganic radioactivity and thus made possible the unambiguous chromatographic identification and estimation of the diphosphates. The use of internal markers, as discussed in the preceding section, supplied the neces-

[2] Synthetic "pGp" was recently given to us by Dr. A. M. Michelson. It did not separate from pAp under our conditions of electrophoresis and chromatography.

TABLE I
EFFECT OF SNAKE VENOM ENZYMES ON TMV-RNA

	Type of RNA-Preparation	Enzymes and Reaction Conditions[a]	Diphosphate Products mole/mole RNA	Exp. 1	Exp. 2	Exp. 3
1	Citrate-bentonite (0.35 mg)	Diesterase (1.5 U), then crude venom (0.5 mg) 24 hr. (pH 9.5, 0.05 M borate)	pCp	0.4	0.37	0.4
			pUp		0.3	
			pAp	0.2	0.31	0.82
			pC > p		0.15	0.16
2	Bentonite (0.5 mg)	Diesterase (0.6 U) (pH 8.8, 0.008 M borate)	pPyp[b]	~0.5		
			pAp	~0.5		
			pC > p	~0.5		
			pU > p	~0.3		
3	No bentonite (0.5 mg)	Diesterase (0.6 U) (pH 8.8, 0.008 M borate)	pCp	20		
			pAp	1.7		
			pUp	6		
			pC > p	60		
			pU > p	45		
4	Citrate-bentonite (0.39 mg)	Diesterase (0.6 U) (pH-stat, pH 9.5)	pPyp[b]	1.3		
			pC > p	2.7		
			pU > p	2.0		
5	Sucrose-gradient peak of bentonite-prep. RNA (0.17 mg)	Diesterase (0.75 U), then crude venom (0.5 mg) 24 hr. (pH 9.5, 0.04 M borate)	pPyp[b]	0.81		
			pAp	0.46		
			pC > p	~0.6		
6	Versene-bentonite (0.38 mg)	Diesterase (1.9 U) (pH 9.5, 0.02 M borate) [contains all markers (total OD = 15)]	pCp	0.7		
			pUp	2.6		
			pAp	1.6		
			pC > p	11.5		
			pU > p	13.5		
7	Sucrose-gradient peak of versene-bentonite-prep. RNA (0.87 mg)	Diesterase (8.3 U) (pH 9.5, 0.02 M borate) [markers (total OD = 8) added *after* digestion]	pCp	0.19		
			pUp	0.18		
			pAp	0.15		
			pC > p	0.65		
			pU > p	0.22		
			pA > p	0.18		

[a] Except where noted, pAp and "pCp" or "pUp" (total OD ~2) were present in each enzyme digestion. The amounts of diphosphates found were corrected for the marker recovery. Digests in which crude venom was used were directly chromatographed (Felix *et al.*, 1960), while diesterase digests required a preliminary purification by electrophoresis. Digestion time was 18–24 hours. [b] pPyp represents material in the area of both "pCp" and "pUp" from chromatograms where these were not well separated.

sary checks on the technique and the required correction factors.

The alternate procedure made use of preliminary electrophoretic separations (pH 7.4 phosphate) of the digests obtained either with purified diesterase or with crude snake venom. The band of material in the nucleoside 2′(3′), 5′-diphosphate and nucleoside 2′3′(cyclic),5′-diphosphate area located between the nucleotides and the inorganic phosphate was cut out, eluted, desalted with Norite, and subjected to two-dimensional chromatography. Four or five distinct, though faint, spots were usually obtained, two or three of which corresponded to the cytidine 2′(3′),5′-diphosphate, uridine 2′(3′),5′-diphosphate, and less frequently the adenosine 3′5′-diphosphate markers. Two others, located between the mononucleotides (see Fig. 2), were identified as the 2′,3′-cyclic derivatives of the pyrimidine 2′(3′),5′-diphosphates.

The amounts and relative proportions of the open P^{32} diphosphates were variable, but frequently they amounted to less than one mole per mole RNA after correction for the recovery of the markers (see Table I). The pyrimidine diphosphates generally predominated greatly. However, 2′3′(cyclic),-5′-diphosphates were also frequently present, with the cyclic cytidine derivative predominating. When the stability of the cyclic pyrimidine diphosphates toward snake venom enzymes was studied, the cytidine was found much more resistant than the uridine derivative to the ring-opening action of the enzyme (Table II). Thus it appears probable that the two observed forms of the diphosphates are of the same origin. The variability in their amounts and proportions speaks in favor of their being derived from artifact chain ends rather than from actual terminal groups.

Many attempts were undertaken to abolish the cause of these artifact chain ends and minimize the danger of 5′-phosphate ester breakage occurring concomitantly with the 3′-ester hydrolysis due to the venom diesterase. It appeared that the spurious hydrolysis was not due to hydroxyl ions, since it appeared to be lessened if digestion was performed at pH 9.5 rather than at pH 8.0. Traces of ribonuclease-like enzymes in the RNA preparations surely contributed to the spurious end groups, since recent techniques leading to more stable RNA of lower protein and enzyme content (Fraenkel-Conrat *et al.*, 1961) also diminished the terminal diphosphates appearing in the digests.

Indications have recently been obtained that the presence of great amounts of markers during the digestion caused the appearance of unusual amounts of material in the diphosphate areas, as illustrated in Table I (preparation 6 *vs.* 7). This may be due to a selective inhibition of the diesterase by the high concentration of diphosphates, which enables traces of 5′-phosphate-splitting nucleases to create more ends. An alternate interpretation of this observation is that phosphate exchange occurred at the level of the cyclic compounds, similar to a phenomenon observed with C^{14}-labeled nucleosides (Sugiyama and Fraenkel-Conrat, 1961). Further

TABLE II

YIELD OF PRODUCTS (%) OBTAINED BY THE ACTION OF SNAKE VENOM ON 2′,3′(CYCLIC),5′-DIPHOSPHATES OF CYTIDINE AND URIDINE[a]

	Products ← 2′(3′),5′-Diphosphate (pXp)	Starting Material 2′,3′(Cyclic),5′-Diphosphate (pX > p)	Products → 2′,3′-Cyclic Phosphate (X > p)	Products → 3′-Nucleotide (Xp)
X = Cytidine				
50 μl venom	32	37	—	30
5 μl venom	—	80	20	—
0.5 μl venom	—	80	20	—
X = Uridine				
50 μl venom	100	0	—	—
5 μl venom	67, 76	17, —	—, —	16, 24
0.5 μl venom	38, 19	45, 67	10, 14	7, —

[a] About 0.2–0.3 μmole (total OD ~3) of each of the cyclized diphosphates treated at 36° for 18 hr. in pH 9.5 borate (about 0.01 M) and 0.003 M magnesium chloride with a centrifuged solution of 10 mg snake venom (*crot. adam.*) per ml in amounts indicated. When uridine 2′(3′),5′-diphosphate was similarly treated with 5 or 0.5 μl of the venom, it was recovered unchanged. The data listed represent percentages of the total recovered absorbancy. Over-all recovery of absorbancy was 55–73% in all of these experiments.

experiments are required to establish the nature of this reaction, but it now appears definitely preferable to perform the digestion in the absence of markers, particularly 2′3′(cyclic),5′-diphosphate markers (*e.g.*, preparation 7). Separate experiments serve to establish the enzyme resistance of the two types of diphosphates. This has generally been found to be similar for both types (over 80%), except that the ring-opening action of the enzyme is highest with adenosine 2′3′(cyclic),5′-diphosphate and lowest with cytidine 2′3′(cyclic),5′-diphosphate (see Table II). The over-all recovery of each pair of markers (pX > p + pXp) after electrophoresis, charcoal adsorption and desorption, and two-dimensional chromatography was about 50%.

A few observations made in the course of these experiments, and which have complicated our task, will now be briefly reported. As stated, two digestion products, cytidine 2′3′(cyclic),5′-diphosphate and uridine 2′3′(cyclic),5′-diphosphate, were detected; they accompanied the nucleoside diphosphates upon electrophoresis, but moved among the nucleotides upon chromatography. Upon hydrolysis with perchloric acid one of these gave uracil as the only base but the other gave two spots, one coinciding with cytosine, the other having an R_F close to that of uracil on methanol-ethanol-HCl chromatograms.[3] Yet, other indications supported the belief that the original material represented the 2′3′(cyclic),5′-diphosphate of cytidine. The 2′3′(cyclic),5′-diphosphates of both pyrimidines were thereupon prepared by cyclizing the open nucleoside 2′(3′),5′-diphosphates with *N,N′*-dicyclohexylcarbodiimide (pXp → pX > p). The cyclized pyrimidine diphosphates coincided with the radioactive materials discussed above. Surprisingly, the cytidine diphosphate, whether cyclized or not, gave upon perchloric acid hydrolysis two spots corresponding to cytosine and similar to uracil, respectively, as had the corresponding radioactive material isolated from RNA digests. The unexpected second component proved to contain phosphorus and appeared to be a cytosine nucleotide by its spectral, electrophoretic, and chromatographic characteristics. It was present only in hydrolysates of the diphosphate (pCp or pC > p) or of a mixture of the 2′(3′)- with the 5′-phosphate (Cp + pC), but not in separate digests of the two components. It was shown that this was not the result of incomplete digestion, since this nucleotide was found even after digestion in sealed tubes at 100° with an excess of perchloric acid and for different time periods. The reaction mechanism responsible for this observation remains to be established.

Two additional pairs of labeled spots were occasionally seen after digestion by snake venom of TMV-RNA. These corresponded in their behavior to 3′-uridylic and 3′-cytidylic acids and to the corresponding 2′,3′-cyclic compounds. They never reached in amounts the cyclic or open diphosphates occurring in the same digests. Since the model experiments on the action of snake venom on the synthetic cyclized diphosphate also gave small amounts of these products under certain conditions, it would appear that they originate from the corresponding cyclic pyrimidine diphosphates (Table II).

Action of Bacterial Phosphomonoesterase on P^{32}-TMV-RNA.—Preliminary experiments indicated that treatment of P^{32}-TMV-RNA (0.5 mg) with amounts corresponding to 10^{-1} to 10^{-4} mg of the purified phosphatase from *Esch. coli* (Garen and Levinthal, 1960) caused rapid release of 0.05 to 0.03% of the phosphorus, corresponding to 3–2 mole/mole RNA without correction for the presence of RNA fragments in the preparation used. Longer incubation periods did not significantly increase the amount of phosphate released. The lower levels of enzyme caused no significant loss of infectivity of the RNA (see Table III).

The technical aspects of the electrophoretic separation and estimation of inorganic phosphate in monoesterase digests are very favorable. In contrast to the nucleotides in alkaline or venom digests, the undegraded RNA does not, upon electrophoresis, diffuse and scatter P^{32} throughout the paper. Thus as little as 20–30 cpm in the area of inorganic phosphate gives a small sharply defined radioautographic spot far from the 10^6 counts of the undegraded RNA (Gordon *et al.*, 1960), and similar-sized blank eluates from in front of or behind that spot may give as little as 1 or 2 cpm. Yet, the reproducibility of very low values ($\lesssim$1 mole/mole) from one experiment to another was sometimes, and unaccountably, poor.

Experiments reported to us by Dr. P. Whitfeld had shown that incubation of TMV in pH 6 citrate

[3] These products were identified through the use of C^{14}-labeled RNA (Sugiyama and Fraenkel-Conrat, 1961).

TABLE III

Esch. coli-PHOSPHOMONOESTERASE TREATMENT OF TMV-RNA

Type of Preparation	Conditions of Enzyme Treatment[a] Substrate-Enzyme Ratio	Time (Min.) (36°)	Phosphate Released (Mole/Mole RNA)	Infectivity[b] (Reconstituted) (% of Control RNA, Reconstituted)
RNA	5	60	2.2, 2.5	0.01
	25	30	2.8	4
	1000	30	2.7	20
	3000	30	2.3	21
Bentonite-RNA	3000	30	2.4	70
	1250	30	2.0	65
	1250	120	1.7	90
	125	30	2.1	93
RNA	67	30	2.8	152
Bentonite-RNA	67	30	2.8	103
Same from citrate-treated virus[c]	67	30	0.9	82
	100	30	0.6	75
	67	30	0.3	68

[a] pH 7.4 Tris buffer (0.005 M) containing 0.001 M Mg^{++} was used for these experiments. [b] The conclusion that phosphomonoesterase does not cause inactivation of TMV-RNA has been arrived at in our previous publication (Gordon *et al.*, 1960) and was confirmed by Schuster (1960). [c] This preparation was also degraded with phosphodiesterase (see Table I, prepn. 1). Several other preparations which were subjected to monoesterase and diesterase treatments also showed little monoesterase susceptible material, and low diphosphate formation with diesterase (*e.g.*, Table I, prepn. 5).

buffer for 18 hours caused release of a small amount of P^{32} from the virus. RNA prepared from such virus by the bentonite procedure (Fraenkel-Conrat *et al.*, 1961) proved identical to other TMV-RNA preparations in all respects except the one under discussion: from most of these RNA preparations very little phosphate was released by the monoesterase. The amounts usually ranged from 0.01–0.005% of the total P and were thus less than one mole per mole RNA (0.015%). Bentonite-prepared RNA without citrate pretreatment of the virus gave amounts of phosphate which ranged from 0.5 to 2.5 mole per mole RNA.

Concurrent experiments were performed in which RNA from typical TMV preparations was fractionated by means of density-gradient centrifugation. With gradients of 10–50% or 2–20% sucrose, 18–24 hours of centrifugation were required to distribute the RNA in a roughly gaussian manner over the top 30–50% of the tube.

The RNA was recovered from the sucrose solution by means of ethanol precipitation. Its concentration was determined by radioactivity counting and often also by spectrophotometry. Groups of tubes corresponding to the leading fraction, the peak, and the trailing material were pooled. In most experiments the bulk of the material upon monoesterase treatment yielded very little phosphate (0.002–0.005%); so did the leading material. In contrast, the trailing material which had sedimented least far in the gradient gave more phosphate upon enzyme treatment than did the starting material. Centrifugation in a sucrose gradient thus appears to fractionate a species of macromolecular RNA lacking monoesterase-susceptible phosphate groups from material relatively rich in such groups and thus presumably of lower chain length. Whether the latter represents only fragments of the

TABLE IV

Esch. Coli-PHOSPHOMONOESTERASE (PME) TREATMENT[a] OF SUCROSE GRADIENT FRACTION OF TMV-RNA

Type of RNA Preparation, and Gradient Fraction Derived from It	Phosphate Released (Mole/Mole RNA)	Infectivity (Reconstituted) PME Treated % of Untreated	Infectivity (Reconstituted) Untreated % of TMV
Bentonite RNA	1.0	100, 112	42
10–50% sucrose[b]:			
peak (55%)	0.46		
trail (4%)	2.6		
Bentonite RNA from versene-pretreated virus	0.41, 0.65	127, 94	36, 55
10–50% sucrose[b]:			
peak (72%)	0.35	70	29
trail (6%)	1.1	104	25
2–20% sucrose[b]:			
peak (86%)[c]	0.36	79	32
trail (5%)	0.7	94	20
Same, different sample	1.7, 1.5	120	29
2–20% sucrose[b]:			
peak (72%)	0.7	118	10
trail (23%)	2.0	48	18
trail end (5%)	8.4	—	0

[a] About 1 μg enzyme per 150 μg RNA, pH 7.4 ammonium acetate buffer (0.003 M), containing 0.001 M Mg^{++}; 30 minutes 37°. In a few comparative experiments, somewhat more phosphate was released in this medium than in the Tris buffer employed in earlier experiments (see Table III). [b] All sucrose used was pretreated with 1/10 volume of 5% bentonite at 0° for 1 hr., then freed of bentonite by ultracentrifugation (40,000 rpm, 2 hr.). Gradient tubes (0.5 × 2 in.) containing 0.5–1.5 mg RNA were centrifuged in a swinging bucket rotor at 35,000 rpm for 18–24 hr. The temperature of the tube contents was 5–10° at the end of the run. [c] This preparation was also degraded with phosphodiesterase (see Table I, experiment 7).

viral RNA or an RNA of different origin has not as yet been determined (see Table IV).

The infectivity of sucrose-gradient-fractionated RNA was associated with the bulk of the RNA, and lower specific infectivities were usually found in the leading and trailing fractions, particularly in the latter if it was rich in monoesterase-susceptible material (Table IV). However, the recovery of infectivity and its stability upon incubation were erratic and frequently low in the sucrose gradient fractions. The reason for this appeared to lie in the presence of nucleases in the sucrose. Thus TMV-RNA was found to lose most of its infectivity upon 24-hour incubation in 20% sucrose, but not if the sucrose had been passed through a dialysis membrane or had been treated with, and freed from, bentonite.

When bentonite-treated sucrose was used in preparing the gradient tubes, the RNA was recovered in seemingly fully active form. Nevertheless, many variables remain to be investigated before the sucrose gradient technique can be proposed as a preparative means for the isolation of highly monodisperse TMV-RNA.

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New Ribonucleic Acid Species Associated with the Formation of the Photosynthetic Apparatus in *Euglena gracilis**

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The conversion of dark-grown *Euglena gracilis* cells, which lack the photosynthetic apparatus, into green organisms under the influence of light is accompanied by a massive synthesis of plastid proteins and a considerable rise in the RNA content of several subcellular fractions. The nucleotide composition of the RNA of the various subcellular fractions was determined, and substantial differences between the ribonucleic acids of homologous fractions from colorless and green cells were recorded. These findings suggest that specific RNA species are produced during the development of chloroplasts.

We have shown in a previous paper (Brawerman and Chargaff, 1959) that the nucleotide composition of the total ribonucleic acid of green *Euglena gracilis* differed significantly from that of the colorless organisms in which chloroplast formation was prevented by the absence of light. In another recent publication (Brawerman *et al.*, in press) we have discussed the evidence that the process through which light induces the formation of chlorophyll-containing plastids in many ways resembles the induced formation of enzymes in micro-organisms. The massive character of the reorganization of the synthetic abilities of the cell leading to the development of chloroplasts made it appear of interest to examine the composition of the ribonucleic acids of various subcellular fractions of *Euglena* cells engaged in what could be considered as a pronounced example of cell differentiation. A brief account of some of the findings has appeared (Brawerman *et al.*, 1961).

EXPERIMENTAL

Cultures.—*Euglena gracilis*, strain z, was grown in the complex medium used before (Brawerman and Chargaff, 1959). Cultures in the stationary phase of growth served for the experiments.

Cell Fractionation.—The washed cell samples were stored at −15° for 2–3 days and then subjected to the fractionation procedure described in a preceding publication (Brawerman *et al.*, in press). The plastid fractions were always collected by centrifugation after removal of the unbroken cells at low speed.

Nucleotide Analysis.—The analytical procedures used have been described before (Brawerman and Chargaff, 1959). The hydrolysis with NaOH was usually performed for 2 days to ensure complete cleavage to nucleotides. Longer incubation at 30° resulted in the extensive deamination of cytidylic acid. The chromatographic separations were all carried out in the customary ammonium isobutyrate solvent. The minor nucleotide fraction moving more slowly than guanylic and cytidylic acids could, in the case of the hydrolysates of the subcellular RNA fractions discussed here, be separated satisfactorily from the other nucleotide components, in contrast to the behavior of the total RNA studied before (Brawerman and Chargaff, 1959). The preparations from the green plastid specimens gave poor chromatograms; but this could be improved by preliminary paper chromatography of the hydrolysate for one day in 1-butanol–0.5 N ammonia (6:1), in which the nucleotides do not move. The preparations from the colorless plastid fractions were, for the sake of uniformity, treated similarly.

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