The Effect of Cycloheximide on RNA Metabolism in Leaves of the Tobacco Plant

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INTRODUCTION

In the previous paper, the authors reported the bipartite nature of the effect of cycloheximide on tobacco mosaic virus (TMV) multiplication in tobacco leaf discs (OUCHI et al., 1969). Cycloheximide inhibited TMV multiplication at concentrations higher than 2.0 ppm, while it enhanced viral multiplication in leaf discs at 1.0 ppm provided that the treatment was initiated 24 hr after inoculation. Treatment before or immediately after inoculation resulted in inhibition even at 1.0 ppm. The phenomenon could be related to the finding that actinomycin D inhibited TMV multiplication when treated in the early phase of infection (SEMAL, 1967).

KERRIDGE (1958) reported that cycloheximide inhibited DNA and protein syntheses in *Saccharomyces carlsbergensis* up to 80—90 per cent in a concentration range of 0.5—1.0 ppm, but did not immediately inhibit the sythesis of RNA. Similar patterns of inhibition of protein synthesis were after-wards dealt with by several authors(SIEGEL et al., 1964; 1965; WIDUCZYNSKY et al., 1965; FUKUHARA, 1965).

HIRAI et al. (1960) found that RNA of a stable type in terms of turnover rate was accumulated in tobacco leaf discs treated with cycloheximide and postulated that stability would be a clue to its accumulation. Similar accumulation of RNA in cycloheximide treated cells had been reported of yeast (SIEGEL et al., 1964), tissue from hypophysectomized rats (FIALA et al., 1965), and onion leaves (SHISHIYAMA et al., 1965). SIEGEL et al. (1964) found that the syntheses of RNA and nucleotides in *Saccharomyces pastorianus* were not retarded in rate in a concentration which caused a complete inhibition of protein synthesis and further showed that the syntheses were even enhanced at a certain concentration.

The enhancement and inhibition of TMV multiplication by cycloheximide led the authors to postulate that protein and RNA metabolisms in the antibiotic treated leaf discs might be drastically changed and hence to characterize by MAK column chromatography the RNA in tobacco leaf discs treated with cycloheximide.

The present communication describes the result of experiments which were aimed at detecting the changes in RNA metabolism in leaf discs treated with cycloheximide, especially in the stimulative or inhibitory concentration.

MATERIAL AND METHOD

Preparation of tobacco leaf discs : Tobacco plant (*Nicotiana tabacum* L. cv. Bright Yellow) were grown in clay pots with sandy soil and supplied with sufficient nutrients, including minor elements. Fully developed leaves of same age were harvested and punched out with a leaf puncher (Kiya Seisakusho, Tokyo) into discs, 10 mm in

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diameter. The discs were mixed throughly in a paper bag and floated on distilled water.

Cycloheximide treatment : The discs were taken at random and transferred onto the surface of either distilled water or cycloheximide solution, 1.0 ppm or 3.0 ppm, in Petri dishes and incubated in a light chamber controlled at 28°C for an appropriate period of time.

Incorporation of ^{32}P *and* ^{14}C-*glycine* : Leaf discs were floated on water or cycloheximide solution containing ^{32}P-orthophosphate or ^{14}C-glycine. ^{32}P-orthophosphate was added to give 1.0 μC or 10.0 μC per ml (carrier free) and ^{14}C-glycine was adjusted at 0.025 μC per ml (specific activity, 5.0 mC/mM).

Quantitative assay of RNA : A modification of Ogur-Rosen method (Ogur et al., 1950) was employed for the extraction of RNA. Fifteen leaf discs were homogenized with 10 ml of 95% ethyl alcohol and the homogenate was centrifuged at 2,800 xg for 20 min. The precipitate was washed with 70% ethyl alcohol containing 0.1% $HClO_4$. The washed precipitate was extracted twice with ethyl alcohol-ether mixture (3 : 1 v/v) at 70°C for 10 min to remove lipids, followed by repeated extraction with 0.2% $HClO_4$. The lipid free precipitate was then incubated with 10 ml of 10% $HClO_4$ at 4°C for 18 hr. The precipitate was further washed five times with 5 ml of 10% $HClO_4$ and the washes were added to the extract (RNA fraction). The fraction was subjected to optical density measurement and orcinol pentose assay. For the measurement of radioactivity, 1 ml of each fraction was added to 10 ml of the solvent consisting of PPO (2, 5-diphenyloxazole) 7.0 g; POPOP (2, 2′-p-phenylene-bis 5-phenyloxazole) 0.025 g ; naphthalene 120 g ; and dioxane 1.0 l. The radioactivity was measured with a Liquid Scintilation Counter, Model SK 4, made by the Nuclear Chicago Co., U.S.A.

Column chromatography of nucleic acids : Nucleic acids were extracted by the procedure of Kubo (1966). Thirty leaf discs were homogenized with 50 ml of cold 1% pyrophosphate and the homogenate was vigorously stirred for 10 min with an equal volume of water-saturated phenol. The mixture was centrifuged at 10,000 xg for 10 min and the water phase was further treated twice with phenol. To the water phase thus obtained was added 2 volumes of cold ethyl alcohol. The precipitated nucleic acids were dissolved in 10 ml of 0.4 M phosphate-buffered saline (pH 6.8) (PBS) and fractionated on a methylated albumin-Kieselguhr (MAK) column. The MAK column was prepared as follows: 15 g of hyflosupercel was suspended in 50 ml of 0.4 M PBS and the suspension was heated to boiling with stirring. To the suspension was added 10 ml of 0.5% methylated albumin (Fraction V of bovine serum albumin) after cooling. The suspension was then charged in a column, 18 mm in diameter and 400 mm in length. The top of the MAK was then covered with 1.0 g hyflospercel suspended in 0.4 M PBS. The column was finally washed with 200 ml of 0.4 M PBS. The nucleic acid preparation was adsorbed on the MAK and the excess was washed with 100 ml of 0.4 M PBS. The adsorbed nucleic acids were eluted with a linear gradient of saline, 0.4 — 1.6 M, at an elution speed of 1 ml per min in a room controlled at 28°C. Each tube received 4 ml of eluate and was subjected to optical density measurement. Radioactivity of each fraction was measured with the automatic Dekatron Scaler, Model E-5, made by Metro Electronics Co., Kyoto.

Ribonuclease (RNase) *activity measurement of leaf homogenate* : Leaf discs incubated for 24 hr either in the presence or absence of cycloheximide were homogenized with 10 ml of 0.1 M PBS (PH 7.2) and the homogenate was centrifuged at 10,000 xg for 30 min. The supernatant was used as enzyme preparation. The substrate employed

was a yeast high molecular weight RNA. The reaction mixture contained 1.0 ml of 1.0% RNA, 2.0 ml of 0.1 M phosphate buffer (pH 7.4) and 1.0 ml of the enzyme preparation and was incubated at 28°C for 30 min. The reaction was stopped by adding an equal volume of cold TCA containing 0.025% uranylacetate. The TCA soluble fraction was subjected to orcinol pentose assay. The substrate efficiency was checked with a commercial pancreatic ribonuclease, 5 x crystallized (Sigma Chemical Co., U.S.A.).

RESULT

1. *RNA Content in Leaf Discs Treated With Cycloheximide*

The RNA content of tobacco leaf leaf discs incubated in the presence of cycloheximide at concentration of 1.0 or 3.0 ppm were determined 12, 24, 36 and 48 hrs respectively after treatment initiation. The relative RNA content in the treated discs was represented in Fig. 1. It is evident that leaf discs treated with cycloheximide contain larger amount of RNA than the control discs. The actual content of RNA in the control discs increased for the first 24 hrs and then decreased to the original quantity, finally falling down to 80 per cent of the original titer by 96 hrs of incubation, while that in cycloheximide treated discs kept the increased level for at least 96 hrs. The result therefore supports the notion that cycloheximide induces an increment of RNA in the treated cells (SIEGEL et al., 1964; FUKUHARA, 1965).

2. *Incorporation of ^{32}P-Orthophosphate and ^{14}C-Glycine Into RNA*

Leaf discs were incubated in cycloheximide solutions containing respective radioisotopes and the incorporation rate of these isotopes into RNA was measured. The result

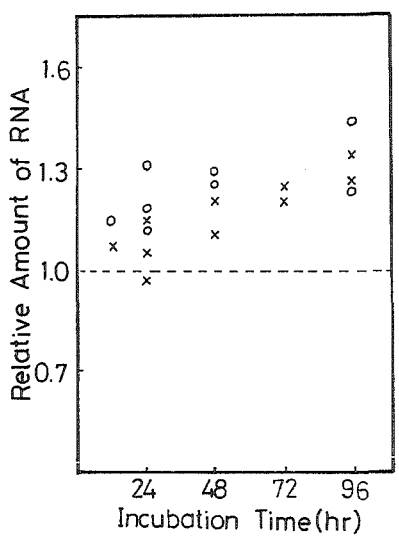

Fig. 1. RNA contents in the leaf discs treated withcycloheximide.
Leaf discs were taken at random and incubated either in distilled water (dotted line), 1.0 ppm cycloheximide (○), or 3.0 ppm cycloheximide(×) for indicated period of time and subjected to RNA extraction. Each plot represents an average of five replicates, 15 leaf discs for a replicate.

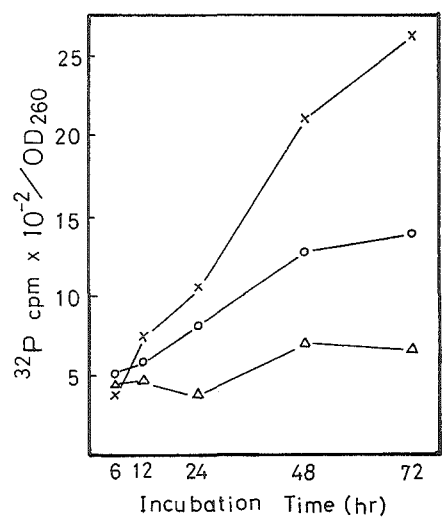

Fig. 2. Efect of cycloheximids on ^{32}P incorporation finto RNA.
Leaf discs were incubated either in water (○), 1.0 ppm (×), or 3.0 ppm (△) cycloheximide, containing respectively 1.0 μC/ml orthophosphate-^{32}P and subjected to RNA extraction.

of ^{32}P incorporation is shown in Fig. 2. It was apparent from repeated experiments that the presence of cycloheximide in the incubation medium causes a slight decrease of ^{32}P incorporationinto RNA at least for the first 6 hrs. The rate of incorporation, however, was accelerated within the next 6 hrs in the leaf discs treated at 1.0 ppm and this accelerated rate was kept almost constant, the relative rate being twice as high as that of control leaf discs. The incorporation rate at 3.0 ppm, however, leveled off by 12 hrs after treatment initiation. This fact seems to contradict the high content of RNA in the discs treated at 3.0 ppm (Fig. 1), but supports the notion the the degradation of RNA *in vivo* is inhibited by cycloheximide (HIRAI et al., 1960). RNA synthesis is, at any rate, accelerated by cycloheximde treatment at 1.0 ppm. This was substantiated by ^{14}C-glycine incorporation into RNA, as shown in Fig. 3. The incorporation rate in this experiment was measured of leaf discs incubated for 3 hrs (3—6 hrs, 9—12 hrs, 21—24 hrs, 45—48 hrs respectively after the start of treatment) in the radioactive medium containing 1.0 ppm cycloheximide. For the first 6 hrs of incubation, no significant difference was observed between the treated and controldiscs. However, the incorporation rate thereafter was always higher in the treated discs, the relative rate being 1.6 times as high as that of the control. The result would then be a conclusive evidence for the enhanced synthesis of RNA in the leaf discs treated at 1.0 ppm and suggests that cycloheximide also stimulated the synthesis of purine bases.

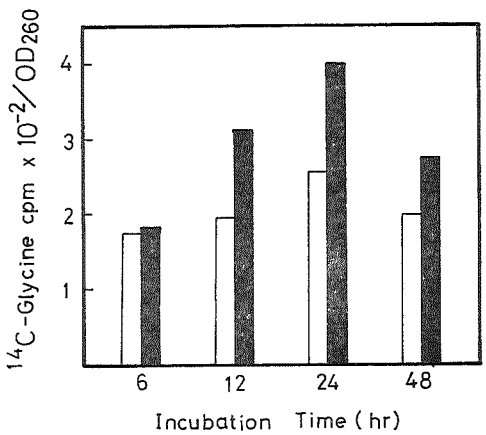

Fig. 3. Enhanced incorporation of glycine-^{14}C into RNA fraction.
Leaf discs were incubated either in water (open bars) or 1.0 ppm cycloheximide (closed bars) and transferred to the radioactive medium 3 hr before the indicated time and incubated for 3 hrs.

3. *Ribonuclease Activity of Leaf Discs Treated With Cycloheximide*

The result shown in Figs. 1, 2, and 3 suggested that the higher content fo RNA in the leaf discs treated at 1.0 ppm could probably be accounted for by the enhanced synthesis of RNA, while RNA in the discs incubated in 3.0 ppm would rather be a simple accumulation due to the inhibition of the degradation process. It is worthwhile then to measure the ribonuclease activity in extracts prepared from these cycloheximide-treated leaf discs.

The result shown in Table 1 clearly indicated that the homogenate of cycloheximide-treated leaf had less ribonuclease activity. The result might not be a direct reflection of the *in vivo* degration, but it at least suggests that the degradation of RNA might be slow in rate in the discs treated with cycloheximide.

4. *Column Chromatography of Nucleic Acids of Tobacco Leaf Discs Treated With Cycloheximide*

To substantiate the above findings on the cycloheximide effect, it is essential to characterize the nucleic acids in the antibiotic treated leaf discs. Nucleic acids adsorbed on the MAK column were eluted with a linear gradient of saline and the result is shown in Fig. 4.

The elution patterns suggest the following : 1) treatment of leaf discs with 3.0 ppm

Table 1. Effect of cycloheximide on ribonlucease activity

Enzyme	Experiment No.	Cycloehximide concentration (ppm)	Relative RNA-se activity (%)*
Homogenate of tobacco leaf discs **	I	0	100
		1.0	63
		3.0	39
	II	0	100
		1.0	69
		3.0	64
	III	0	100
		1.0	68
		3.0	52
Commercial pancreatic RNA-se ***		0	100
		1.0	128
		3.0	96

* $\frac{\text{Activity in extracts from treated discs}}{\text{Activity in extracts from untreated discs}} \times 100$, average of 3 replicates, 5 samples in a replicate.

** Leaf discs were incubated for 24 hrs.

*** Ribonuclease I, 5 × crystalized (Sigma Chemical Co., U.S.A.), 0.1 μg/ml.

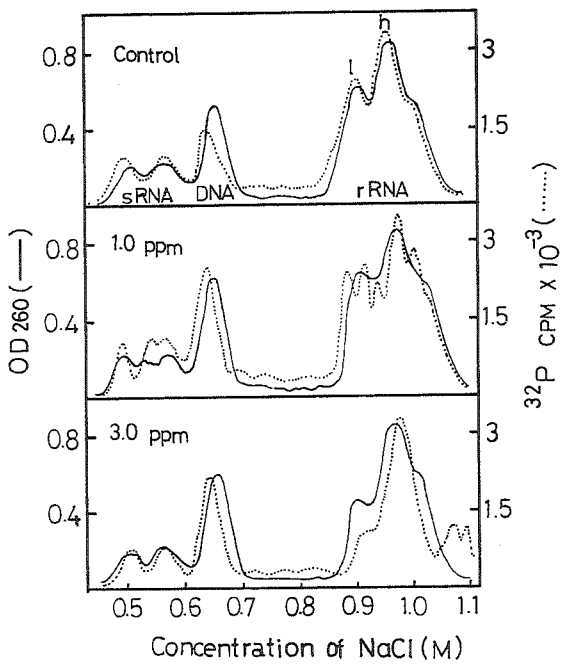

Fig. 4. MAK column chromatograms of nucleic acids extracted from the tobacco leaf discs treated with cycloheximide. Leaf discs were incubated for 24 hrs in water (cont.), 1.0 ppm, or 3.0 ppm cycloheximide, containing respectively 1.0 μC/ml or thophosphate-^{32}P and subjected to RNA extraction. Optical density (solid line) and radioactivity (dotted line) were measured of each fraction.

cycloheximide leads to the reduction of the light ribosomal RNA as perceptible in optical density plots, while treatment at 1.0 ppm does not have a profound effect on

the light rRNA as far as the optical density profile is concerned; 2) this finding is supported by the patterns of ^{32}P incorporation, namely that incorporation into light rRNA is definitely reduced by the treatment at 3.0 ppm, but not so by the treatment at 1.0 ppm. The incorporation pattern with the leaf discs treated at 1.0 ppm indicates that cycloheximide gives rise to an abnormal synthesis of RNA; 3) since the elution profiles were obtained of the total nucleic acids, it is possible that the synthesis of both cytoplasmic ribosomal RNAs is inhibited by cycloheximide but that of chloroplastic ribosomal RNA is not.

The decrease in the incorporation of ^{32}P into RNA (Fig. 2) was further characterized by MAK chromatography. Fractionation was run of nucleic acids extracted from the leaf discs which had been subjected to ^{32}P incorporation for a short period of time. Fig. 5 summarizes elution profiles of these nucleic acids. The result clearly shows

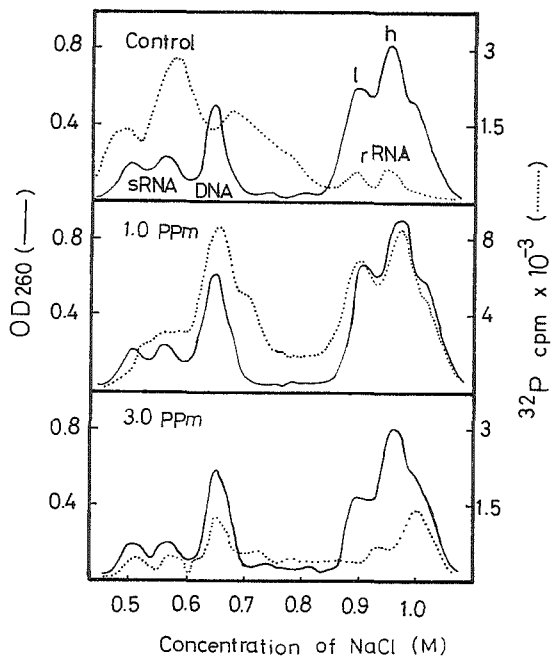

Fig. 5. Elution profile of total nucleic acids extracted from leaf discs subjected to short period labelling.
Leaf discs incubated for 24 hrs either in water (cont.), 1.0 ppm, or 3.0 ppm cycloheximide were transferred to the radioactive medium (10 μC/ml) containing respective concentration of the antibiotic and incubated for 1 hr. Nucleic acids extracted from these discs were fractionated on MAK column.

that the incorporation into total nucleic acids was higher in the discs treated at 1.0 ppm and lower in those treated at 3.0 ppm than the control discs, justifying the findings in Fig. 2. It is also apparent that ^{32}P incorporation into rRNA was extremely low in the control discs while that in the 1.0 ppm treated discs was found to be almost complete in terms of elution profile. The result would then be a conclusive evidence for the notion that RNA synthesis is enhanced by the cycloheximide treatment at 1.0 ppm.

Elution profile of nucleic acids extracted from the discs treated at 3.0 ppm, on the other hand, suggests that the inhibitory effect of the antibiotic is rather selective, as evident in the lower total incorporation and higher incorporation rate of the fractions eluted at around 1.0 M NaCl comparing to the control nucleic acids.

The authors then focussed their attention on the process of abolition of the light rRNA. The result is represented in Fig. 6. It is explicitly shown that the light rRNA partially disappear from the elution profile by 24 hrs after treatment initiation and continues to gradually disappear, leading to a complete absence of the peak after 60 hrs of incubation. Fig. 7 represents an elution pattern of ribosomal RNA of the

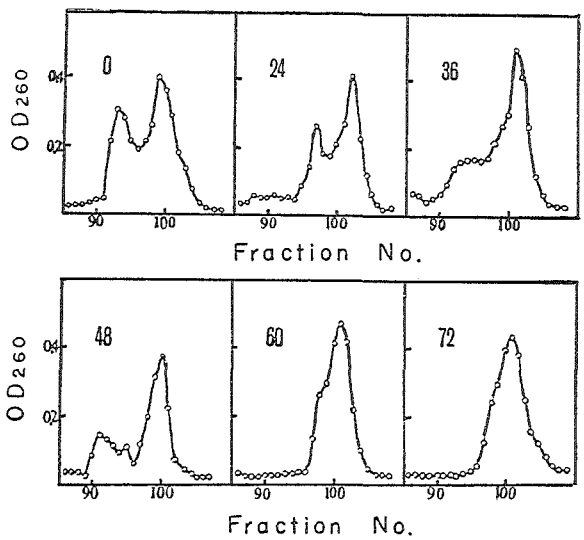

Fig. 6. Sequential changes in optical density profiles of ribosomal RNA of the leaf discs inicubated in 3.0 ppm cycloheximide solution for 0, 24, 36, 48, 60, and 72 hrs.

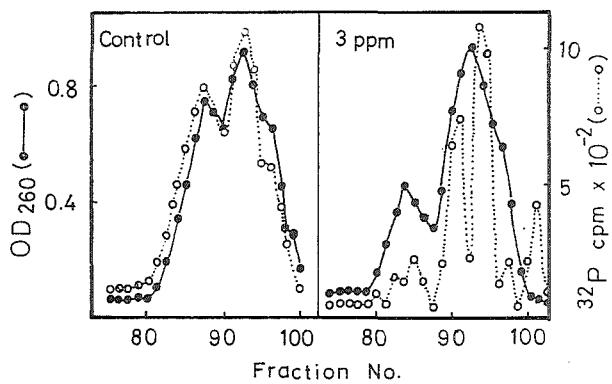

Fig. 7. Elution pattern of ribosomal RNA extracted from the leaf discs which had been incubated for 18 hrs in 3.0 ppm cycloheximide. Optical density (solid line) and radioactivity (dotted line) were measured of each fraction.

leaf discs incubated for 18 hrs in 3.0 ppm cycloheximide solution. The optical density plots are rather close to that of the control, but the ^{32}P incorporation pattern shows that the effect had already become noticeable at this stage. It should also be noted that incorporation into the heavy rRNA is also disturbed.

The result of a similar experiment carried out with leaf discs treated at 1.0 ppm suggested that there was no critical effect on the light rRNA on the basis of the optical density profile.

In view of these chromatograms, the inhibition of TMV multiplication in the presence of 3.0 ppm of cycloheximide could probably be related to the iintegrity of the light rRNA.

DISCUSSION

Cycloheximide has been generally accepted to be an inhibitor of protein synthesis, although the primary site for its action has been a subject of discussion (SISLER et al., 1967; SISLER, 1969).

Quantitative assays of RNA in the leaf discs incubated in 1.0 or 3.0 ppm cycloheximide solution (Fig. 1) agree with published findings (FIALA et al., 1965; HIRAI et al., 1960; OUCHI et al., 1969; SHISHIYAMA et al., 1965; SIEGEL et al., 1964). The stability against degradation might be partially due to a lower activity of ribonuclease in the treated leaf, as was illustrated in Table 1. Incorporation of ^{32}P into RNA, however, showed that the cycloheximide effect is bipartite at the level of RNA synthesis, i. e., inhibitory at 3.0 ppm and stimulative at 1.0 ppm (Fig. 2). Therefore the higher content of RNA in the leaf discs treated at 1.0 ppm should be substantially different from that in discs incubated in 3.0 ppm solution, i. e., the former is mainly accounted for by stimulated synthesis and the latter is mostly due to the inhibition of degradation.

Incorporation of ^{14}C-glycine into RNA supports this notion. ^{14}C-glycine incorporation into RNA was always high in the leaf discs treated at 1.0 ppm compared to that of the control, suggesting that cycloheximide at this concentration stimulates not only the synthesis of RNA but also the syntheses of puring and probably pyrimidine bases. SIEGEL et al. (1964) found in *Saccharomyces pastorianus* that cycloheximide stimulated the incorporation of ^{32}P into nucleotides and RNA. WIDUCZYNSKY et al. (1965) also reported a higher incorporation of ^{14}C-adenine into RNA of *Saccharomyces ellipsoideus*. Therefore the stimulation of RNA synthesis seems to be a rather common property of cycloheximide in a certain concentration range. Cycloheximide, however, acts as an inhibitor of RNA synthesis in other concentration ranges, as was shown in Fig. 2. The authors assume that these action patterns would probably account for the bipartite nature of the effect of cycloheximide on TMV multication.

The above findings, however, could not, by themselves, explain the mechanism of the cycloheximide effect, because the qualitative aspect of RNA was not included. Therefore the authors made a chromatographic analysis of nucleic acids with MAK column and explicitly elucidated that cycloheximide at 3.0 ppm has a profound effect on the structural unit of ribosome, leading to the almost complete abolition of the light ribosomal RNA. Incubation in 1.0 ppm, however, did not have such a drastic effect on the light rRNA, though some abnormal synthesis of ribosomal RNA was suggested by the incorporation pattern of ^{32}P. FUKUHARA (1965) reported a similar effect on RNA synthesis in *Saccharomyces cerevisiae*. Research pertinent to this line was done by FIALA et al. (1965) with *Neurospora crassa* and De KLOET (1965) with

Saccharomyces carlsbergensis.

In the light of recent findings on the characteristics of chloroplast ribosomes and ribosomal RNA (BOADMAN et al., 1965; DYER et al., 1968; LYTTLETON, 1962; STUTZ et al., 1967), the elution profiles of the total RNA extracted from the cycloheximide treated discs (Figs. 4 and 5), moreover, raised an interesting question whether the antibiotic selectively inhibits the synthesis of cytoplasmic ribosomal RNA or it also blocks the synthesis of chloroplast ribosomal RNA. Works in this aspect are under way.

On the basis of these results, the authors assume that the previously reported inhibition of TMV multiplication at 3.0 ppm (OUCHI et al., 1969) would probably be due to the absence of the light rRNA as a unit of integrity, although the reasons for the disappearance of the light rRNA peak from the chromatogram should be sought further before coming to a conclusion. The undulate pattern of ^{32}P incorporation into heavy rRNA in the discs treated at 1.0 ppm suggests that cycloheximide affects not only the rate of synthesis but also the structural integrity of RNA. At the moment, therefore, the authors consider that the enhancement of TMV multiplication might be a result of shifting the stimulated nucleotide and RNA syntheses toward the program of viral RNA synthesis.

SUMMARY

The effect of cycloheximide on RNA metabolism in tobacco leaf discs was analyzed to signify its inhibitive or stimulative effect on TMV multiplication. RNA content increased in the leaf discs incubated in 1.0 or 3.0 ppm solution. ^{32}P-incorporation into RNA, however, was accelerated at 1.0 ppm and disturbed at 3.0 ppm, suggesting that the increment of RNA in the leaf discs treated at 1.0 ppm is qualitatively different from that in the discs incubated at 3.0 ppm, i. e., the former is the result of stimulated synthesis while the latter is mainly due to the inhibition of degradation. Column chromatograms with methylated albumin Kiselguhr clearly elucidated that incubation of discs in 3.0 ppm led to the abolition of the integrity of the light ribosomal RNA. The change in chromatographic pattern was discernible 24 hr after the initiation of treatment of leaf discs. Incubation in 1.0 ppm cycloheximide, however, did not give rise to a drastic change in chromatograms, although an undulate incorporation of ^{32}P-orthophosphate into ribosomal RNAs was noticed. ^{14}C-glycine incorporation into RNA suggested that cycloheximide not only enhances RNA synthesis but also stimulates the synthesis of purine bases. Ribonuclease activity of leaf homogenate remarkably decreased by cycloheximide treatment, suggesting that the accumulation of RNA in the discs treated at 3.0 ppm could be acounted for by the retardation in RNA degradation. The inhibitory and stimulative effects of cycloheximide on TMV multiplication were discussed on the basis of these results.

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シクロヘキシミドのタバコ葉核酸代謝におよぼす影響

大内成志・古沢 巌・赤井重恭

シクロヘキシミドはその濃度により，タバコ葉中における TMV 増殖を促進または阻害する．その機構を明らかにするために，これら増殖阻害（3.0ppm）または促進（1.0ppm）濃度で処理したタバコ葉の核酸代謝について研究した．シクロヘキシミド処理タバコ葉においては，いずれの濃度においてもRNAの含量が高くなる．^{32}Pおよび^{14}C-グリシンのとりこみ実験から，1.0ppm処理葉のRNA増加は合成促進によるものであり，3.0ppm区のRNAは分解速度の低下による蓄積に基づくものと考えた．これらの濃度で処理した葉の核酸代謝は質的にも異なる．3.0ppm処理区においては ℓ-rRNA のピークが低下するとともに，^{32}P のとりこみも低下しており，リボゾーム構造に著しい変化がもたらされたものと考えられる．1.0ppm処理区においては ℓ-rRNA の変化は認められないが，rRNAへの^{32}pのとりこみが著しく促進する．しかし，とりこみのパターンは必らずしも均一でなく，シクロヘキシミドの核酸代謝におよぼす影響が複雑であることを示している．これらの結果から，3.0ppm における TMV 増殖阻害効果はリボゾーム機能の破壊によるものであり，1.0ppm 処理による増殖促進効果は，シクロヘキシミドにより高揚された核酸代謝をウイルス増殖プログラムに転換するものであろうと考えた．