Changes in Ribonucleic Acids during Uredospore Differentiation

L. Ramakrishnan and Richard C. Staples

Boyce Thompson Institute, Yonkers, New York 10701.

Supported in part by Grant GB-5492X from the U.S. National Science Foundation.

Accepted for publication 18 February 1970.

ABSTRACT

Uredospores of the bean rust fungus (*Uromyces phaseoli*) contain the same complement of nucleic acids throughout germination and differentiation that is found in resting spores. The amount of template activity of the RNA increases during differentiation at the time of formation of the appressorium, then declines. Incorporation of uridine into template RNA occurred only during differentiation of the germ tube, which suggests that formation of the infection structures may depend on synthesis of messenger RNA. Phytopathology 60:1087-1091.

Additional key words: Bean rust spores, template RNA, appressoria.

The biochemical events of germination and differentiation of obligately parasitic fungi are poorly understood. Previous work (17) has suggested that spores of the rust fungi depend upon protein synthesis for germination, which probably is guided by a stored messenger RNA (10). Involvement of a long-lived messenger RNA has been implicated in the germination of conidia of *Peronospora tabacina,* a fungus which is obligately parasitic on tobacco (4).

Differentiation of uredospores seems to depend on the synthesis of a new messenger RNA as judged by the sensitivity of differentiating wheat rust uredospores to actinomycin D (3). The nuclear divisions which occur during differentiation (8) also suggest that nucleic acid metabolism is substantially altered during appressorial formation. A template RNA having many of the properties of a messenger was previously described in resting uredospores of the bean rust fungus (10), and the present work was begun to study the fate of this presumed messenger RNA during germination and differentiation. Additional studies were concerned with changes in ribosomal and transfer RNA.

MATERIALS AND METHODS.—Uredospores of the bean rust fungus (*Uromyces phaseoli* [Pers.] Wint.) were grown and collected weekly from infected leaves of bean (*Phaseolus vulgaris* L. 'Pinto') plants grown in controlled environment chambers as described previously (12). After harvest, spores were stored for 4 days in a stoppered bottle at 4 C.

Spore germination.—Spores were prepared for germination by floating them on water in a cold room at 4 C for 16 hr (18). After hydration, the spores were dusted onto baking dishes containing collodion membranes using the procedure described by Wynn & Gajdusek (16). Paraffin oil was used to induce the formation of appressoria, and at least 90% of the germ tubes were differentiated. Germination was carried out in the dark at 20 C.

Preparation of ribosomes.—Spores germinated on membranes were ground with sand in a mortar or pulverized in liquid N. It was preferable to use sand when preparing polyribosomes, but liquid N was preferred for transferase assays. The grinding buffer (11) contained 0.45 M sucrose, 0.05 M Tris [tris(hydroxymethyl) amino methane] buffer, pH 7.5, 5×10^{-3} M $MgCl_2$ and 5×10^{-3} M 2-mercaptoethanol. The homogenate was centrifuged for 15 min at 20,000 *g*, and stored at −20 C. The thawed supernatant was centrifuged at 300,000 *g* for 30 min to obtain the ribosomal pellet. The pellet was resuspended in a suitable volume of suspension buffer (11) (0.02 M Tris, 5×10^{-3} M $MgCl_2$, 0.015 M KCl, and 5×10^{-3} M 2-mercaptoethanol), and centrifuged at 13,000 rpm for 10 min to remove aggregates. When required, the ribosomes in the pellet of aggregates were released by adding 5 μg/ml of trypsin to the suspension and incubating on ice for 1 hr.

Preparation of radioactive RNA.—After dusting the collodion membranes with hydrated spores, the membrane surface was sprayed with water as usual. Then 1 ml of water containing 50 μc of uridine-5-^{3}H (specific activity 25.2 c/mmole) was sprayed directly on the membrane surface, and the spores germinated as before. The RNA was extracted by pulverizing 20 membranes in liquid N. As thawing occurred, 20 ml of redistilled phenol (88%) were added together with 10 ml Tris, pH 7.5, and 2 ml of sodium lauryl sulfate (SLS), and the RNA was extracted as described previously (10). Radioactivity in the RNA was determined by the procedure of Trewavas (14) in which the RNA was precipitated by cetyltrimethylammonium bromide, collected on glass fiber filters, and counted by liquid scintillation.

Template assay of RNA.—The template assays were carried out using a supernatant (S 30) from *Escherichia coli* cells depleted of messenger RNA (mRNA) as described previously (10).

Density gradient analysis.—The RNA was fractionated on a 5-20% sucrose gradient as previously described (10). Ribosomes were fractionated on a 15-30% gradient according to Staples et al. (12).

Transferase assay.—Transfer assays were carried out as previously described by Yaniv & Staples (17). When required, the supernatant was pre-incubated according to Nirenberg (9). The reaction was carried out for 30 min at 30 C.

MAK column chromatography.—Chromatography of RNA on columns of methylated albumin kiesulguhr (MAK) was carried out as described previously (10). A linear sodium chloride gradient of 0.2 M to 1.5 M was used for elution. The RNA was precipitated from the pooled fractions by adding 1 mg of yeast tRNA as carrier, 0.15 M potassium acetate, and two volumes of absolute ethanol.

Polyribosome depletion.—Polyribosomes were puri-

TABLE 1. The recovery of RNA from nondifferentiated or differentiated bean rust uredospores germinated on collodion membranes

Hr of germination	RNA recovered mg/g spore	
	Nondifferentiated	Differentiated
4	6.03	7.33
8	7.38	5.22
24	5.05	4.02
Avg	6.15	5.52

Each value is an avg of two determinations of one preparation of spores. The avg yield of RNA from both differentiated and nondifferentiated spores was 5.83 ± 1.2 mg RNA/g spore.

fied on a step gradient as described previously (12), and depleted of messenger RNA by dispersing them in 2 ml of suspension buffer, then dialyzed 4 hr against 0.1 M Tris buffer, pH 7.8, 0.05 M NaCl, and 1×10^{-4} M $MgCl_2$ at 4 C. The pellet was resuspended in dialyzing buffer, centrifuged for 1 hr at 65,000 rpm and the pellet dissolved in Tris-KCl buffer (0.01 M Tris, 2×10^{-2} M KCl, pH 7.8). RNA was extracted from the polyribosomes by making the suspension 0.5% with respect to SLS, incubating a few min at room temp, and deproteinizing with phenol (88%).

RESULTS.—*General properties of RNA.—Unfractionated RNA.*—Estimation of total RNA extracted from germinated uredospores by the phenol-SLS procedure revealed that the amount of RNA does not change greatly during germination (Table 1). Results of analyses varied only about 18% from the average value of 5.83 mg/g spores, regardless of the stage of germination or differentiation.

Fractionation of RNA.—RNA from resting spores was isolated and characterized previously (10), and the RNA from germinated spores was found to be similar. When analyzed on a sucrose gradient (Fig. 1-A), RNA from germinated spores (4 hr) was resolved into three principal fractions having sedimentation constants of 4.6, 19, and 27 S which were previously shown (10) to be tRNA and the two ribosomal RNAs. Though not resolved here, DNA occasionally appeared as a shoulder on the light ribosomal RNA (19 S). The RNA was also characterized using MAK columns (Fig. 2), and by polyacrylamide gel electrophoresis (7, 15). Typical profiles were obtained which showed each of the components.

Template activity of RNA.—General.—It was shown previously that dormant uredospores of the bean rust fungus contain a template RNA which stimulates amino acid incorporation in a cell-free system prepared from *E. coli* (10). Consequently, unfractionated RNA prepared from germinating spores was tested for its template activity using the same procedure (Fig. 1-B). During 24 hr of germination, the template activity of the RNA from nondifferentiated spores steadily declined. In contrast, the template activity of RNA from differentiating spores increased briefly between 4 and 8 hr. This suggests that some new template RNA was synthesized during the time when the germ tube differentiated to form appressoria, a process which occurs approximately 4 hr after germination begins (16).

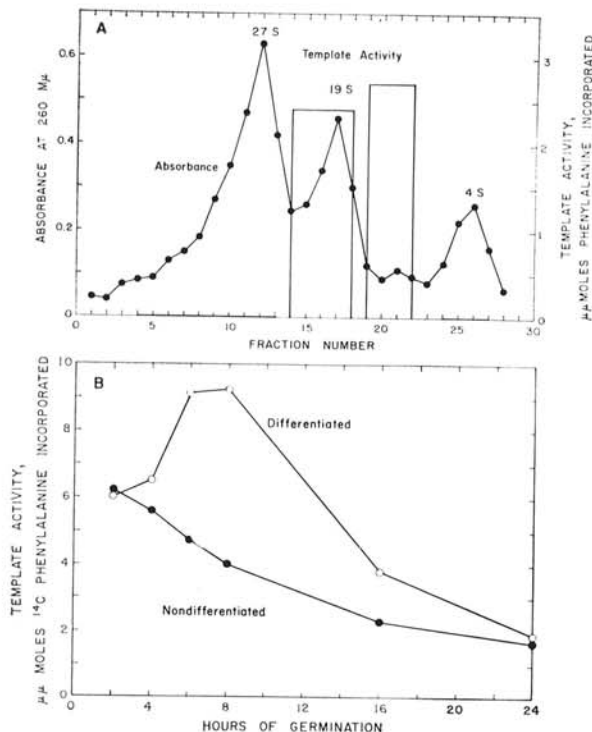

Fig. 1. Template activity of RNA from germinated uredospores of *Uromyces phaseoli*. **A)** Template activity of RNA from spores differentiated for 4 hr after fractionation on a sucrose gradient. The RNA (0.4 mg) was layered on a 5-20% sucrose gradient and centrifuged for 2.5 hr at 65,000 rpm. The fractions indicated by the bars were pooled, RNA-precipitated, and the RNA tested for template activity. The bars represent template activity of the RNA in μμmoles of ^{14}C-phenylalanine incorporated into protein after deducting for the control, which was 3.7 μμmoles. The closed circles represent absorbancy at 260 mμ. **B)** Template activity of RNA from germinated spores. The RNA was extracted and tested for template activity as described in MATERIALS AND METHODS. The values represent μμmoles of ^{14}C-phenylalanine incorporated into protein after deducting the control, which was 3.8 μμmoles.

Resolution of the template activity.—As was shown for resting spores (10), the template activity of RNA from germinated spores was principally located in the 4-19 S region of sucrose gradients (Fig. 1-A).

Template activity of RNA from polyribosomes.—Staples et al. (13) have shown that spores germinated for 4 hr contain at least 60% of their ribosomes as polyribosomes, and template activity should be detectable in RNA prepared from them. When RNA was extracted from polyribosomes isolated from germinated spores (4 hr), it was found to have 3.7 times more template activity than RNA prepared from polyribosomes treated to deplete them of messenger RNA (Table 2). During the preincubation treatment, the ribosomes lost 92% of their capacity to transfer amino acids from charged tRNA unless polyuridylic acid was added as a messenger RNA, showing that messenger RNA was removed from the ribosomes during the depletion treatment.

TABLE 2. Template activity of RNA extracted from polyribosomes of uredospores of *Uromyces phaseoli* differentiated for 4 hr[a]

Preparation	^{14}C-phenylalanine incorporated, cpm
+Polyuridylic acid (40 μg)	21,656
+RNA from polyribosomes (100 μg)	4,297
+RNA from stripped polyribosomes (100 μg)	1,155

[a] Polyribosomes were purified on a step gradient as described previously (12), and depleted of messenger RNA by incubating them in 2 ml of suspension buffer (MATERIALS AND METHODS). The values are corrected for the 2,695 cpm incorporated in the absence of added RNA.

Uridine incorporation into RNA.—General.—^{3}H-uridine was incorporated into RNA at a constant rate throughout 24 hr of germination (13). The incorporation by differentiating spores was 3 times greater than it was for nondifferentiating spores.

In order to study the synthesis of template RNA during differentiation, germinating spores were exposed to ^{3}H-uridine between the 4th to 5th hr (Fig. 3-A) and between the 12th to 13th hr (Fig. 3-B). After exposure, the RNA was fractionated on sucrose density gradients and the patterns compared. These 1-hour periods were chosen, because formation of the appressorium begins around the 4th hr and is complete around the 8th hr (16).

Kinetic studies using density gradients.—RNA from differentiating spores labeled between the 4th and 5th hr was most abundantly labeled in the 4-19 S region of the gradient, while labeling was nearly absent in this region when the RNA was obtained from nondifferentiating spores (Fig. 3-A). In contrast, RNA from germinating spores labeled between the 12th and 13th hr after germination had nearly the same pattern of labeling regardless of whether the spores were differentiated or not (Fig. 3-B). This suggests that template RNA is synthesized in differentiating spores between the 4th and 5th hr after germination, but not between the 12th and 13th hr. This conclusion agrees with the analyses for template activity which showed that this activity increased in differentiating spores until the 6th hr (Fig. 1-B).

A summary of the data from the pulse-labeling experiments shows that 27% of the total radioactivity was distributed in the 4-19 S region when the RNA was obtained from differentiated spores (Table 3). This compares to 13% when the spores were nondifferentiated, and 12-15% when the spores were labeled in the 12th to 13th hr.

MAK analyses.—Reports of many studies have shown that during chromatography on MAK, mRNA elutes during and after the heavy ribosomal RNA (5). RNA prepared from differentiated spores contained a large fraction of radioactivity which eluted from a column of MAK after the ribosomal RNA (Fig. 2-A). This fraction was largely absent when the RNA was prepared from nondifferentiated spores (Fig. 2-B). It was shown previously that the same postribosomal fraction of RNA from resting spores contained much of the template activity of RNA (10). This suggests again that an important fraction of RNA is synthesized between 4 and 8 hr by differentiated spores that is not synthesized by nondifferentiated spores, and which has the characteristics of a messenger RNA.

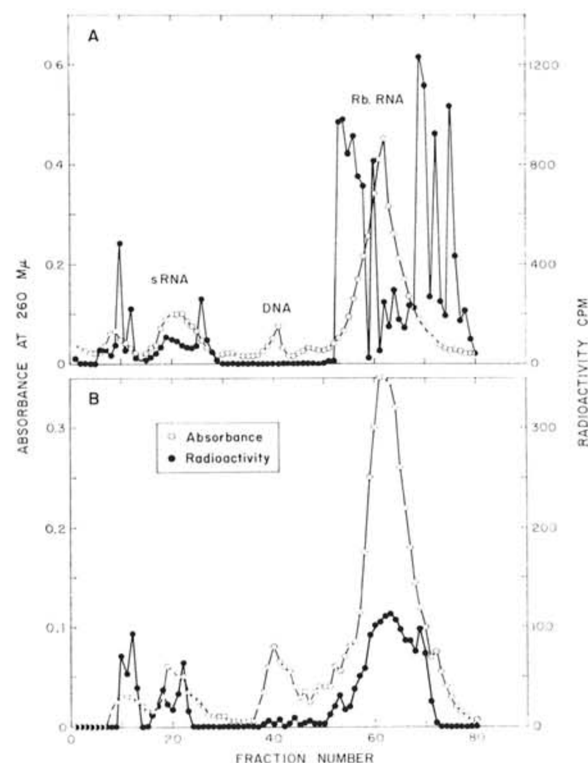

Fig. 2. Column chromatography on methylated albumin kieselguhr (MAK) of pulse-labeled RNA from germinating spores of *Uromyces phaseoli*. Germinated spores were exposed to ^{3}H-uridine for 1 hr between the 4th and 5th hr after germination, and the RNA extracted (see MATERIALS AND METHODS). The RNA was fractionated on a column of MAK, and the fractions were determined for absorbancy at 260 mμ and radioactivity. **A)** Differentiated spores; **B)** nondifferentiated spores.

Analyses of polyribosomes.—The results just described suggest that template RNA is synthesized in

TABLE 3. Percentage distribution of radioactivity in RNA from spores of *Uromyces phaseoli* incubated with ^{3}H-uridine at different stages of germination as determined by sucrose density-gradient centrifugation[a]

	Spores which were			
	Differentiated		Nondifferentiated	
	and labeled during			
RNA species	4-5 hr	12-13 hr	4-5 hr	12-13 hr
Ribosomal (28 S)	31	42	42	41
Ribosomal (19 S)	23	19	20	27
Template (4-19 S)	27	15	13	12
Transfer (4 S)	19	24	24	20

[a] Spores were germinated and pulse-labeled with ^{3}H-uridine for 1 hr from the 4th to 5th hr after germination and from the 12th to 13th hr after germination. Spores which were 4 days old were used for the experiment.

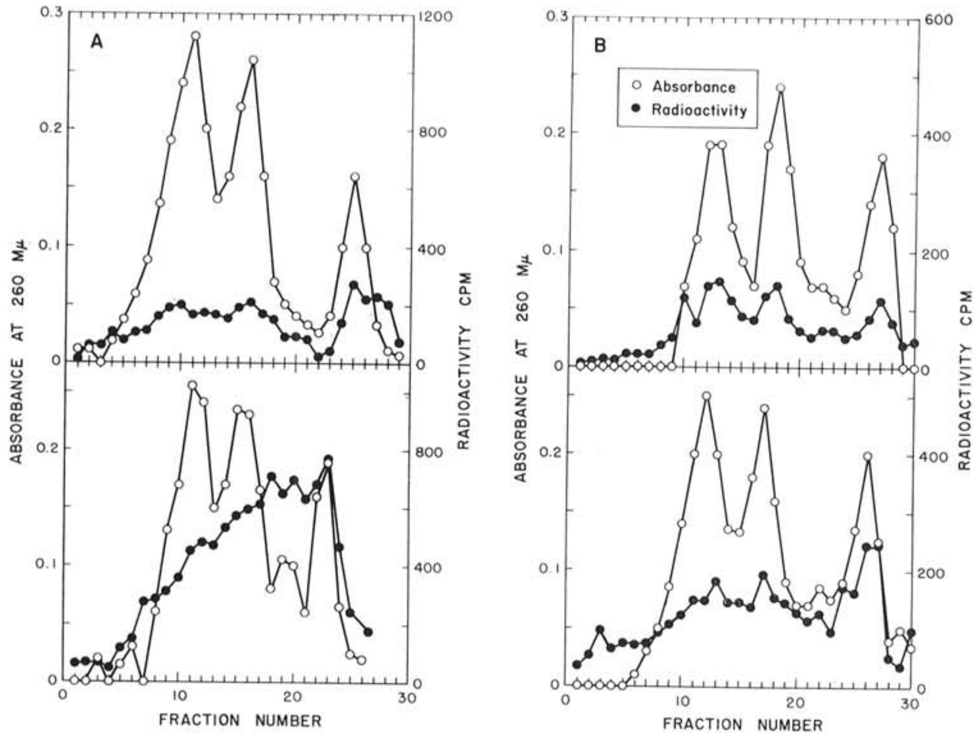

Fig. 3. Density-gradient analyses of pulse-labeled RNA from germinated spores of *Uromyces phaseoli*. Germinated spores were exposed to ^{3}H-uridine for 1 hr **A)** after 4 hr of germination; **B)** after 12 hr of germination, the absorbancy and radioactivity of the fractions determined. Upper curves, nondifferentiated spores; lower curves, differentiated spores.

differentiating spores during formation of the infection structures. To provide further evidence of this, polyribosomes were extracted from differentiated germ tubes which had been labeled for 1 hr with ^{3}H-uridine between the 4th and 5th hr. After fractionation on a sucrose gradient, it was found that a trace of ribonuclease converted most of the polyribosomes to monoribosomes as seen by the large increase in absorbance in the monoribosome area (Fig. 4-A). The radioactivity profile, on the other hand (Fig. 4-B), did not increase in the monoribosome area, even though the radioactivity in the polyribosome area declined. Such results might be expected if the uridine had been principally incorporated into the mRNA attached to the ribosomes, while the ribosomes themselves were largely unlabeled.

Discussion.—Germination of uredospores consists of at least four physiological phases, including a complex fourth phase in which the germ tube differentiates into an appressorium and vesicle (2) accompanied by nuclear division (8). Recently, Dunkle et al. (3) reported that differentiation of wheat rust uredospores is blocked by actinomycin D, an inhibitor of RNA polymerase in many organisms. These results were confirmed in the present studies with the bean rust fungus using 100 ppm of the drug (*unpublished data*), and the results suggest that formation of infection structures often regarded as necessary for infection (1) may accompany or depend upon synthesis of a messenger RNA.

A template RNA in resting bean rust uredospores was recently described which had many of the properties of a messenger (10). Results from the present study demonstrate that the template activity of RNA increases in uredospores triggered to differentiate (Fig. 1-B), suggesting that messenger RNA is synthesized during formation of infection structures. For this reason, it was interesting to find that uridine was incorporated into template RNA only during formation of the appressoria, and not after differentiation was completed (Fig. 3). In fact, only template RNA may be extensively synthesized, even though uridine was also incorporated into ribosomal and transfer RNA (Fig. 3). It was found that polyribosomes isolated from spores exposed to uridine during appressorium formation were radioactive, but the tritium removed by treatment with ribonuclease did not sediment with monoribosomes released by the enzyme (Fig. 4). Apparently, differentiation stimulates synthesis of messenger RNA which then binds with ribosomes, most of which are already present in the spore.

The fact that the synthesis of template RNA ceased toward the end of differentiation and declined in senescing germ tubes offers a clear view of a biochemical role for the host in supporting the growth of this obligate parasite. One role for the host must be its stimulation of a continued synthesis of messenger RNA if growth of the fungus is to continue. Previously, it was reported that transfer activity of ribosomes from germinating spores declines because initiation of new

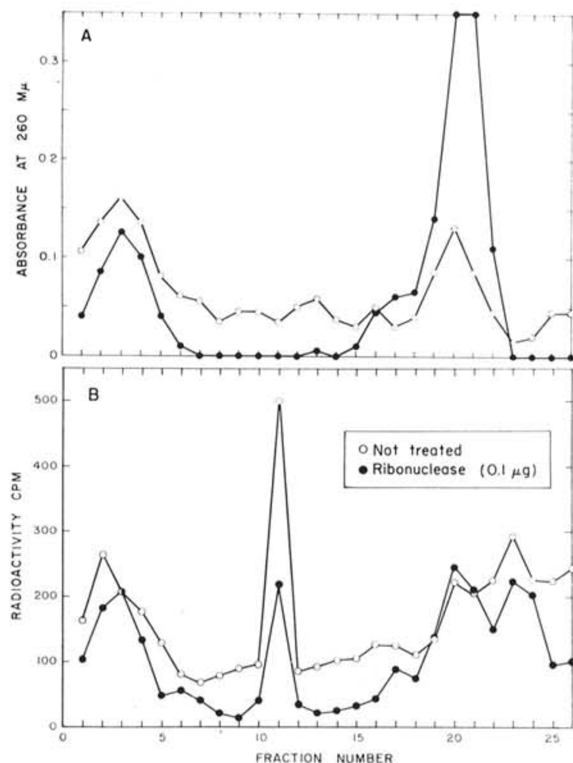

Fig. 4. Effect of ribonuclease on pulse-labeled polyribosomes from uredospores of *Uromyces phaseoli*. The differentiated spores were germinated 4 hr, then pulse-labeled for 1 hr with ^{3}H-uridine and polyribosomes isolated as described in MATERIALS AND METHODS. The ribosomes were layered on a 15-30% sucrose gradient and the radioactivity and absorbancy of the fractions were determined. **A)** Absorbancy profile; **B)** radioactivity profile. Fraction No. 20 contained the monoribosomes.

protein chains declines (18). Now it appears that chain initiation may cease, because, as in other systems (6), messenger RNA becomes limiting. Other possibilities exist, including a decline in activity of the initiation enzymes.

Sterile spores could not be used in these studies, and the influence of bacteria on the labeling patterns had to be considered. Although these experiments may have suffered from a low level of contamination, high amounts of bacteria were apparently not encountered. For example, numerous experiments with uridine in the presence of 1,000 units of penicillin always gave the same results as when penicillin was omitted. Furthermore, the clear difference between the patterns of incorporation by differentiated and nondifferentiated spores (Fig. 3) that were germinated under the same conditions requires some other explanation than simple contamination, especially in view of the fact that the differences at 4 hr (Fig. 3-A) entirely disappeared when cultures 6 hr older were used (Fig. 3-B). Finally, it would be expected that the template activity of the RNA would have increased as germination proceeded if contamination had been involved, whereas it actually declined over 24 hr of germ tube elongation (Fig. 1-B).

LITERATURE CITED

1. CHAKRAVARTI, B. P. 1966. Attempts to alter infection processes and aggressiveness of *Puccinia graminis* var. *tritici*. Phytopathology 56:223-229.
2. DICKINSON, S. 1949. Studies in the physiology of obligate parasitism. II. The behaviour of the germ-tubes of certain rusts in contact with various membranes. Ann. Bot. (London) n. s. 13:219-236.
3. DUNKLE, L. D., R. MAHESHWARI, & P. J. ALLEN. 1969. Infection structures from rust urediospores: effect of RNA and protein synthesis inhibitors. Science 163:481-482.
4. HOLLOMON, D. W. 1969. Biochemistry of germination of *Peronospora tabacina* (Adam) conidia: evidence for the existence of stable messenger RNA. J. Gen. Microbiol. 55:267-274.
5. INGLE, J., J. L. KEY, & R. E. HOLM. 1965. Demonstration and characterization of a DNA-like RNA in excised plant tissue. J. Mol. Biol. 11:730-746.
6. KLEPPE, R. K., & A. P. NYGAARD. 1969. Messenger RNA concentration and protein synthesis during T4 infection of *Escherichia coli* B. Biochim. Biophys. Acta 190:202-210.
7. LOENING, U. E. 1967. The fractionation of high-molecular-weight ribonucleic acid by polyacrylamide-gel electrophoresis. Biochem. J. 102:251-257.
8. MAHESHWARI, R., A. C. HILDEBRANDT, & P. J. ALLEN. 1967. The cytology of infection structure development in urediospore germ tubes of *Uromyces phaseoli* var. *typica* (Pers.) Wint. Can. J. Bot. 45:447-450.
9. NIRENBERG, M. W. 1963. Cell-free protein synthesis directed by messenger RNA. Methods Enzymol. 6: 17-23.
10. RAMAKRISHNAN, L., & R. C. STAPLES. 1970. Evidence for a preformed template RNA in resting uredospores of the bean rust fungus. Contrib. Boyce Thompson Inst. 24:197-202.
11. STAPLES, R. C., A. A. APP, W. J. MCCARTHY, & M. M. GEROSA. 1966. Some properties of ribosomes from uredospores of the bean rust fungus. Contrib. Boyce Thompson Inst. 23:159-164.
12. STAPLES, R. C., D. BEDIGIAN, & P. H. WILLIAMS. 1968. Evidence for polysomes in extracts of bean rust uredospores. Phytopathology 58:151-154.
13. STAPLES, R. C., Z. YANIV, L. RAMAKRISHNAN, & J. LIPETZ. 1970. Properties of ribosomes from germinating uredospores. *In*: Morphological and related biochemical events in host-parasite interaction (in press).
14. TREWAVAS, A. 1967. A new method for counting labeled nucleic acids by liquid scintillation. Anal. Biochem. 21:324-329.
15. WEINBERG, R. A., U. LOENING, M. WILLEMS, & S. PENMAN. 1967. Acrylamide gel electrophoresis of HeLa cell nucleolar RNA. Nat. Acad. Sci. USA Proc. 58:1088-1095.
16. WYNN, W. K., & C. GAJDUSEK. 1968. Metabolism of glucomannan-protein during germination of bean rust spores. Contrib. Boyce Thompson Inst. 24:123-138.
17. YANIV, Z., & R. C. STAPLES. 1968. Study of the transfer of amino acids from aminoacyl transfer ribonucleic acid into protein by ribosomes from uredospores of the bean rust fungus. Contrib. Boyce Thompson Inst. 24:103-108.
18. YANIV, Z., & R. C. STAPLES. 1969. Transfer activity of ribosomes from germinating uredospores. Contrib. Boyce Thompson Inst. 24:157-163.