QUANTITATIVE AND QUALITATIVE COMPARISONS OF POLYRIBOSOMES FROM HEALTHY AND SOUTHERN BEAN MOSAIC VIRUS-INFECTED CONTENDER BEAN

by

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QUANTITATIVE AND QUALITATIVE COMPARISONS OF POLYRIBOSOMES FROM HEALTHY AND SOUTHERN BEAN MOSAIC VIRUS-INFECTED CONTENDER BEAN

INTRODUCTION

The protein synthesis activity associated with polyribosomes in plants is dependent on the availability of mRNA and various protein factors and the ability of the ribosomes to participate in protein synthesis [39]. The concentration of ribosomes occuring as polyribosomes has been directly correlated with the level of *in vitro* incorporation of amino acids into protein by the isolated ribosome preparations [23, 30]. Quantitative changes observed in plant ribosomes following viral infection depend on the plant species, plant age, and virus involved. Increases in the quantity of ribosomes isolated from Mung bean hypocotyls were demonstrated following infection by tobacco mosaic virus [21]. Venekamp and Taborsky [34] showed that potato virus X induces greater ribosome increases in tobacco plants than in potato plants. Decreases in polyribosomes were found in *Nicotiana glutinosa* leaves infected systemically with lettuce necrotic yellows virus [24, 25].

Previous methods used to isolate ribosomes from maturing plant tissue did not provide high polyribosome yields and possessed ribonuclease activity. New methods prevent polyribosome losses caused by aggregation and ribonucleases during extraction [12], thus permitting a study of the proportion of ribosomes occuring as polyribosomes.

This report describes a study conducted on southern bean mosaic virus (SBMV)-infected Contender beans (*Phaseolus vulgaris* L.) to determine what effect viral infection might have on the quantity of polyribosomes and on the qualitative aspects of their participation in protein synthesis.

MATERIALS AND METHODS

Virus production

Southern bean mosaic virus (ATCC culture AC 17) was increased in *Phaseolus vulgaris* L. cv. Bountiful bean grown under green-house conditions. Four weeks post-inoculation (P. I.), leaves were harvested and stored at -20° C in plastic bags. The virus was purified by the chloroform-butanol procedure of Steere [28] except that 1% 2-mercaptoethanol was added to the initial extraction buffer (0.1 M potassium phosphate, pH 7.0). Purified virus suspended in 0.02 M buffer was diluted to 0.4 mg/ml and stored at -20° C until used.

Assay of infectivity

Virus infectivity was determined by local lesion assay on the primary leaves of Pinto bean [22].

Isolation of polyribosomes

Contender beans grown in pots of autoclaved soil were dusted with 600 mesh carborundum and were inoculated 1-2 days after the primary leaves had expanded. An aliquot of SBMV (0.4 mg/ml) was rubbed on each leaf with a cotton swab. Control plants were inoculated with phosphate buffer only. The beans were grown in a growth chamber (14 h photoperiod; average light intensity of 19 400 lx at primary leaf height; 75% relative humidity). Plants were fertilized daily with Hoagland nutrient solution [10].

Polyribosomes were prepared from Contender bean by using a modification of procedures described by Jackson and Larkins [12]. All stages of the procedure were performed at 2-4° C. Freshly harvested bean leaves (2 g) with the midribs excised were homogenized with a Willems Polytron PT 12 20

(3.4 setting) for one min in 20 ml of polyribosome extraction buffer (200 mM Tris-HCl, pH 8.0, 400 mM KCl, 200 mM sucrose, 35 mM $MgCl_2$, and 10 mM ethylenediamine tetraacetic acid (EDTA). The homogenate was centrifuged for 15 min at 30 000 g and the pellet was discarded. The supernatant was layered on a 8 ml sucrose pad (1.75 M sucrose, 40 mM Tris-HCl, pH 8.0, 200 mM KCl, 30 mM $MgCl_2$, and 5 mM EDTA) and centrifuged for 2 h at 192 000 g. The polyribosome pellet was resuspended in 0.4 ml of sterile water or resuspension buffer (40 mM Tris-HCl, pH 8.0, 200 mM KCl, 30 mM $MgCl_2$, and 2 mM EDTA). Concentrations of resuspended polyribosomes were measured at 260 and 280 nm and then stored at -65° C.

Portions of resuspended polyribosomes were layered on 15-45% sucrose gradients. The gradients were formed by layering 1.9, 3.6, 3.6, and 1.9 ml of 150, 250, 350, and 450 mg sucrose/ml, respectively, in gradient buffer (40 mM Tris-HCl, pH 8.0, 20 mM KCl, and 10 mM $MgCl_2$). The gradients were equilibrated for at least 18 h prior to use. Samples were centrifuged for 2.5 h at 283 000 g and 4° C. Gradients were scanned at 254 nm with an ISCO model D gradient fractionator and UA-2 ultraviolet analyzer coupled to an external recorder. Peaks on the chart paper were quantitated with a planimeter. Samples to be assayed for infectivity were either collected from the effluent as gradients were being scanned or were collected from the punctured bottom of a tube containing a duplicate sample of the one being scanned.

RNA and protein assays

RNA extractions were made by grinding 1 g of leaves in 10 ml of GPS buffer (0.1 M glycine, 0.3 M NaCl, 50 mM K_2HPO_4, pH 9.5) [7]. The extract was centrifuged for 15 min at 30 000 g and an aliquot of the supernatant

was layered on a 0-30% log-linear sucrose gradient containing GPS buffer [4]. The gradients were centrifuged for 7.5 h at 283 000 g and 15° C, then scanned and quantitated as above.

Buffer-soluble protein was precipitated in 5% trichloroacetic acid, resuspended in 1.0 N NaOH, and measured by the method of Lowry *et al.* [18].

Preparation of wheat germ extracts

Wheat germ extracts for *in vitro* protein synthesis were obtained using modified procedures of Shih and Kaesburg [27]. Raw wheat germ samples were a gift from the Wall-Rogalsky Milling Co., McPherson, KS. Extracts were prepared at 0-4° C. Wheat germ (3 g) was ground dry for 30 s in a chilled mortar with 3 g of neutralized, acid-washed sand. The powdered germ was mixed with 5 ml of buffer (1 mM Mg acetate, 2 mM $CaCl_2$, 50 mM KCl, 0.5 mM dithiothreitol [DTT], 20 mM Tris-acetic acid, pH 7.6). The resulting paste was then centrifuged at 23 000 g for 10 min. Two to three ml of supernatant were removed, taking care to avoid the upper lipid layer. This supernatant, designated S 23, was centrifuged a second time and then dialyzed for 3 h at 4° C in 1000 volumes of 25 mM Tris-acetic acid, pH 8.0, 90 mM K acetate, 4.5 mM Mg acetate, 0.5 mM DTT. When wheat germ supernatant lacking wheat ribosomes (S 100) was needed, the S 23 extract was centrifuged 2 h at 269 000 g and the upper 75% of the supernatant was saved and dialyzed as above.

Preparation of viral RNA

Viral RNAs were prepared from purified virus by heating the preparation in sodium dodecyl sulfate (SDS)-EDTA buffer followed by phenol extraction and ethanol precipitation as described by Bruening *et al.* [6]. The RNAs

were stored at -65° C until used.

In vitro *protein synthesis*

Reactions mixtures for protein synthesis in a final volume of 100 ul contained: 15 ul of wheat germ extract, 25 mM Tris (pH 8.0), 88 mM K acetate, 2.5 mM Mg acetate, 0.5 mM DTT, 1 mM ATP, 40 ug/ml creatine phosphokinase, 0.2 mM GTP, 5 mM creatine phosphate, 30 uM of each of 19 amino acids, and 0.15 uCi of L-[^{14}C]-leucine (310 uCi/mmole). Assays were run in duplicate at 23° C for 2 h [40] and stopped by the addition of 1 ml of 10% trichloroacetic acid (TCA). After 15 min on ice, the samples were heated to 75-85° C for 15 min, and then cooled in ice water. The TCA-insoluble precipitates were collected on glass fiber filters (Reeve Angel No 934 AH), dried, and counted in 3 ml of scintillation cocktail.

Preparation of bean monoribosomes

To obtain bean monoribosomes functional in the wheat germ system, approximately 90 A_{260} units of bean polyribosomes were converted to 80 S monoribosomes by incubation *in vitro* in a reaction mixture containing 450 ul of S 100 and the same concentrations of the other ingredients used in the *in vitro* protein synthesis system except that unlabeled 30 uM L-leucine was substituted for L-[^{14}C]-leucine. The reaction mixture was incubated at 25° C for 1.5 h and centrifuged at 269 000 g for 2 h. The pellet was resuspended in water, the concentration was determined, and the preparation was stored at -65° C.

Polyacrylamide gel electrophoresis of labeled proteins

The *in vitro* ^{14}C-labeled translation products were analyzed by SDS-polyacrylamide gel electrophoresis by the methods of Weber and Osborne [36] using 10% acrylamide gels in 0.1% sodium dodecyl sulfate (SDS), 0.1% 2-mercaptoethanol, and 0.1 M $NaPO_4$, pH 7.2. The *in vitro* incorporation samples were treated by a modification of procedures by Mayo *et al.*[20]. Samples were treated with 10 mM EDTA, 10 ug/ml RNAse A, 3 mM $CaCl_2$, and 10 mM leucine. Following digestion for 30 min at 30° C, the samples were made 6% in 2-mercaptoethanol, 1 mg/ml in iodacetamide [8], 0.05 M in DTT, 2% in SDS, and 2 ug/ml in pyronin Y [9]. The samples were heated to 60° C for 5 min, cooled, and made 15% in sucrose. Protein standards suspended in 25 mM Tris (pH 8.0), 88 mM K acetate, 2.5 mM Mg acetate, and 0.5 mM DTT were treated the same as above. Proteins used for standards were: bovine serum albumin (MW=67 000), ovalbumin (MW=45 000), southern bean mosaic virus coat protein (MW=29 000), trypsin (MW=23 000), brome mosaic virus coat protein (20 000), and tobacco mosaic virus coat protein (17 500).

After electrophoresis for 4 h at 6 ma/gel, the gels were stained for 12 h in 0.25% coomassie brilliant blue in 45% methanol, 45% water, and 10% acetic acid [1]. Following destaining, the standard gels were scanned at 553 nm on a Beckman Acta III spectrophotometer. A standard curve was prepared by plotting the relative migration of the standards versus the log of their molecular weights. Gels of *in vitro* products were cut into 2 mm slices, incubated overnight at 35° C with 3% Protosol (New England Nuclear) in aqueous toluene scintillation cocktail and counted after cooling to room temperature. Electrophoretic mobilities of the ^{14}C-labeled proteins were compared to those of the protein standards.

RESULTS

Symptom development

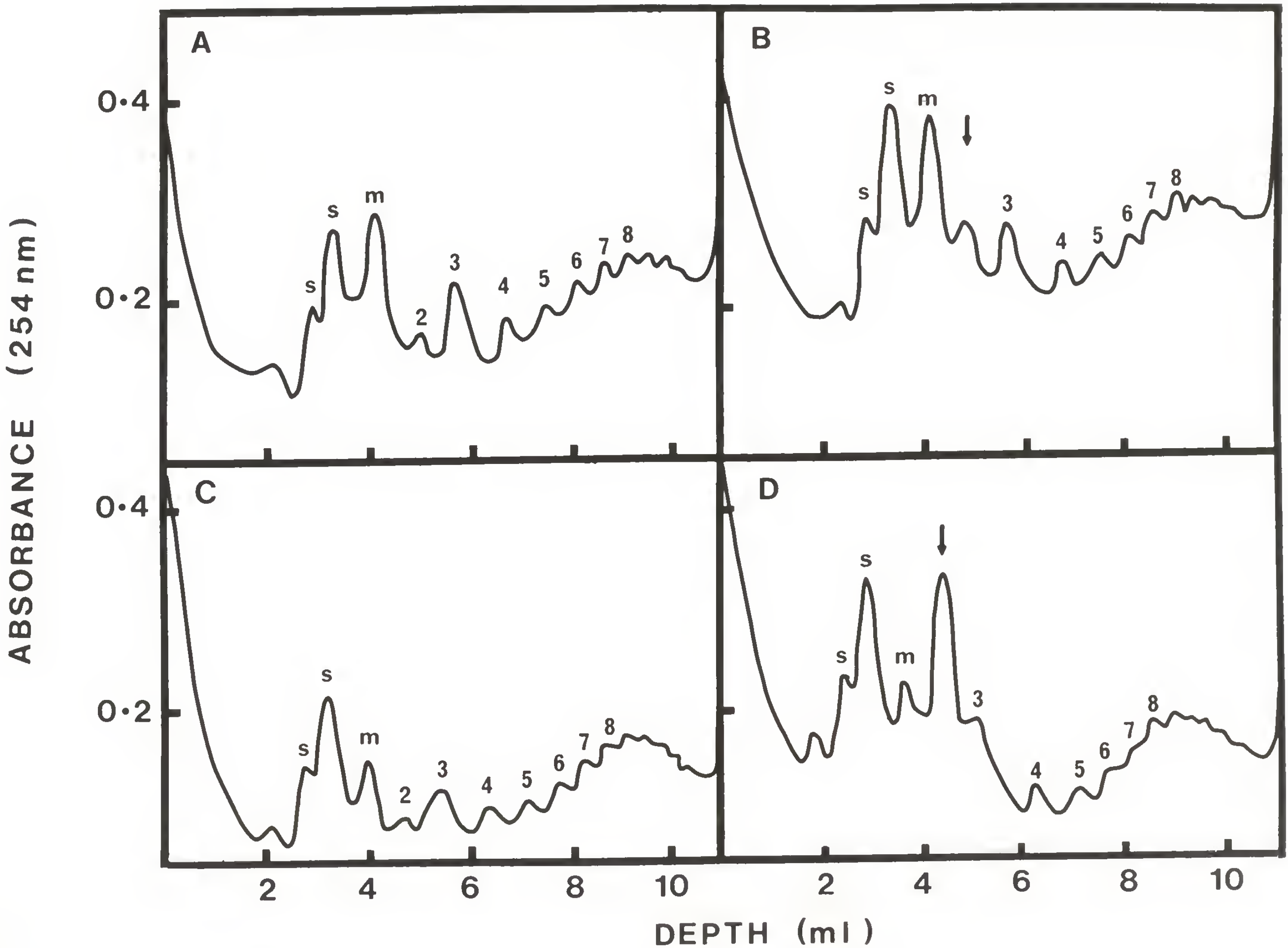
Virus symptoms were expressed on inoculated primary leaves as a very mild vein yellowing at 5-6 days P.I. Viral infection did not stunt the primary leaves. The first systemically-infected trifoliolate leaves to appear 7-8 days P.I. resembled healthy trifoliolates but were about 10% narrower at 15-20 days P.I. The second and third sets of infected trifoliolates emerged 1-2 days later than comparable healthy leaves and were curled, had dark green puckered areas, and were severely stunted.

Polyribosome concentration and size distribution

The relative concentrations and size ditributions of cytoplasmic polyribosomes in healthy and SBMV-infected plants were studied in extracts taken from the inoculated primary leaves and systemically infected trifoliolate leaves of Contender bean. The resuspended polyribosomes had an $A_{260/280}$ of 1.7 to 1.9 depending upon the amount of heavy polyribosomes present. Yields of total ribosomes from primary and trifoliolate leaves varied from 0.05-0.7 mg/g leaves, based on $E_{260}^{1\%} = 110$ [11]. Sucrose gradient RNA preparations from the polyribosomes indicated that 95% of the total rRNA was 18 S and 28 S RNA.

Fig. 1 shows representative polyribosome profiles taken from one of three separate time course studies for healthy and SBMV-infected primary and trifoliolate leaves. Profiles obtained 6 days P.I. or later from infected leaves contain SBMV virions which sediment at a position in the profile corresponding to a small dimer peak in the healthy profiles. To compensate for the UV absorbance contributed by the SBMV particles in the gradient, the area under the SBMV peak in profiles from infected leaves

Fig. 1. Ultraviolet absorption profiles of polyribosomes isolated from primary and trifoliolate leaves of Contender bean. Polyribosomes were isolated from: (a) healthy primary leaves 6 day P.I.; (b) SBMV-infected primary leaves 6 day P.I.; (c) healthy trifoliolate leaves 14 day P.I.; and (d) SBMV-infected trifoliolate leaves 14 day P.I. Ribosome subunits are designated S, monoribosomes M, and polymerized ribosomes as 2, 3, 4, 5, 6, 7, & 8. The 115 S SBMV virion peak is indicated by the arrow.


A
B
C
D
ABSORBANCE (254 nm)
0·4
0·2
DEPTH (ml)
2
4
6
8
10
s
m
3
4
5
6
7
8

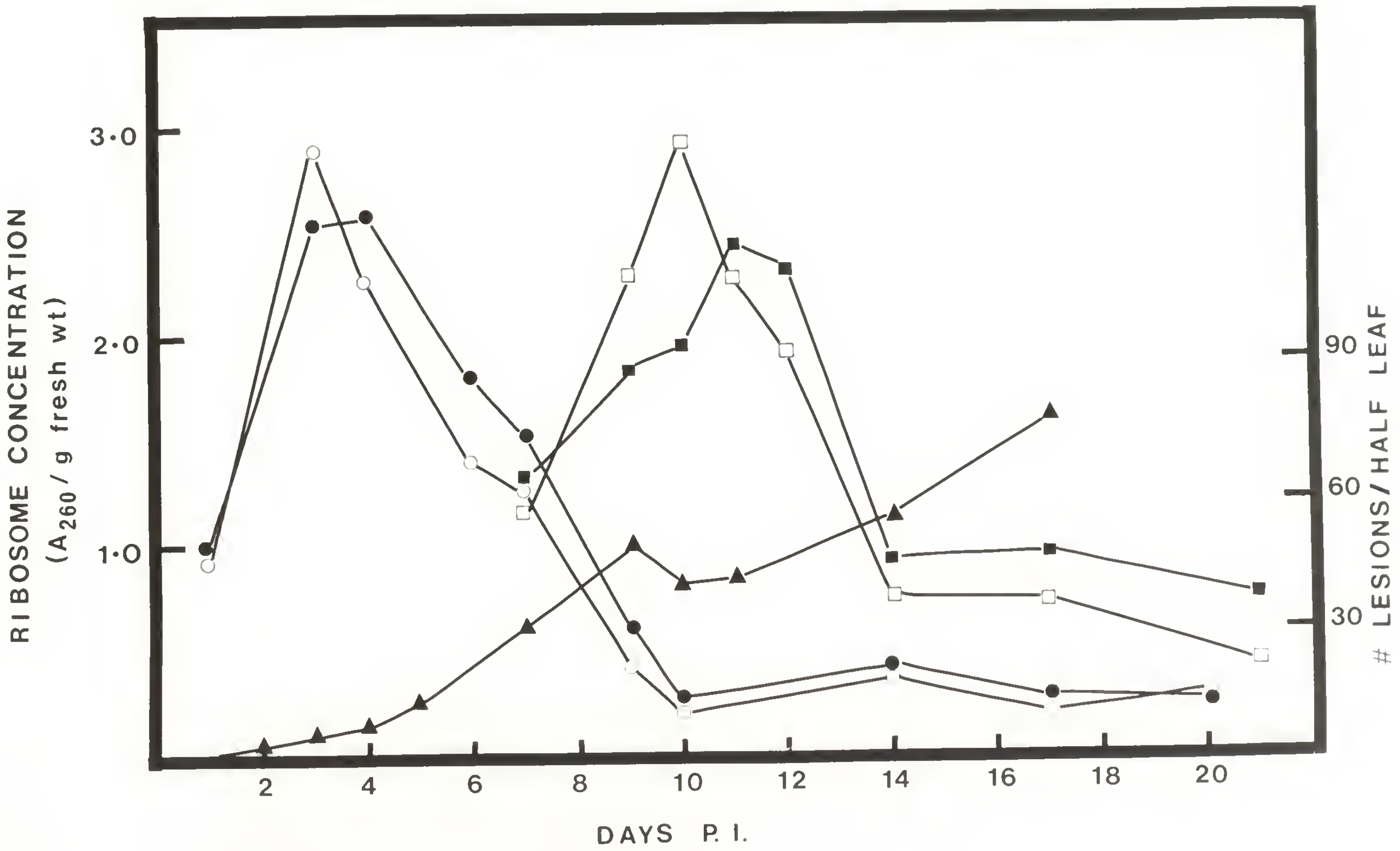
was measured and subtracted from the total absorbance to give an accurate estimate of the total amount of ribosomal material present in the profile.

Fig. 2 shows total amounts of ribosomes recovered from healthy and SBMV-infected primary and trifoliolate leaves. In healthy primary leaves, the total ribosome concentration is maximal at 3 days P.I. and decreases significantly to a constant level by 9-10 days P.I. In infected primary leaves, the total ribosome concentration consistently maintains a level at 5-8 days P.I. which is 20 to 30% higher than that of healthy leaves in all three experiments. At 10 days P.I. and thereafter, the total ribosome concentration in infected leaves is decreasing to a lower level similar to healthy leaves with variations of 10 to 20%. During the 4-9 day P.I. period, SBMV increases rapidly in the primary leaves. Assays of the total buffer-soluble protein of healthy and infected primary leaves indicate a 20 to 60% increase in protein in infected primary leaves during the 6-12 day P.I. period (data not shown).

In the first trifoliolate leaves (Fig. 2), the concentrations of ribosomes in infected leaves were found consistently to be lower at 8-10 days P.I. but after 11 days P.I., the relative levels of ribosomes remained higher in the infected trifoliolate leaves. In all experiments, the maximum levels of total ribosomes in infected trifoliolate leaves were never observed to equal or exceed the maximum levels for the healthy trifoliolate leaves.

Total ribosome concentrations declined in both healthy and SBMV-infected leaves. However, the ratio of polyribosomes to monoribosomes consistently decreased slightly faster in both primary and trifoliolate SBMV-infected leaves (data not shown).

Fig. 2. Changes in the concentration of total ribosomes from: (○) healthy primary leaves; (●) SBMV inoculated primary leaves; (□) healthy trifoliolate leaves; and (■) SBMV systemically infected trifoliolate leaves. Concentrations of SBMV in inoculated primary leaves (▲) were assayed on Pinto bean.


RIBOSOME CONCENTRATION
(A_{260}/g fresh wt)
3·0
2·0
1·0
LESIONS/HALF LEAF
90
60
30
2
4
6
8
10
12
14
16
18
20
DAYS P.I.

Ribosomal RNA concentrations

The GPS extracts of total rRNA contained 18 S and 28 S rRNAs of the free cytoplasmic ribosomes and 16 S and 24 S rRNAs of membrane-bound chloroplast ribosomes. Complete separation of the rRNAs was not obtained, thus the peak areas were combined as 16-18 S rRNAs and 24-28 S rRNAs for planimetry measurements. Comparisons of extracts of total rRNA of the inoculated primary leaves indicate differences similar to those found in the polyribosome profiles (Table 1). Infected leaves contained 20-30% more total rRNA than healthy leaves at 6-10 days P.I. with the major differences in the 24-28 S rRNAs.

Infectivity of polyribosome gradient fractions

Local lesion assays of polyribosome gradient fractions from infected leaves indicated the presence of SBMV virions at 2-3 days P.I. These virions sedimented at the 115 S position of the gradient. Lower amounts of infectivity occurred throughout the gradient fractions of ribosome subunits and heavy polyribosomes at 4 days P.I. and thereafter. Infectivity of the heavy polyribosomes could indicate either polyribosomal-associated viral RNA or aggregated SBMV virions. Purified SBMV fractionated in polyribosome type gradients indicated that infectivity was present in the ribosomal subunit region and the heavy polyribosome region of the gradient, suggesting that aggregated SBMV virions or subviral particles [5] may be present in the fractionated polyribosomes from infected tissue.

In vitro *translation of SBMV RNA*

With SBMV RNA as mRNA, extracts of wheat germ displayed optimum activity at pH 8.0 using 15 ul of S 23 extract per 100 ul of reaction mixture.

Table 1

CHANGES IN RIBOSOMAL RNA CONTENT DURING INFECTION
OF PRIMARY LEAVES

Days P.I.	(Relative A_{260}/ml/g fresh wt)		Ratio of total rRNA
	Healthy	Infected	Infected/Healthy
2	5.38	5.70	1.06
4	4.05	4.20	1.04
6	2.75	3.73	1.35
8	3.18	4.22	1.33
10	3.23	3.65	1.13
12	3.36	3.71	1.10
17	2.53	2.52	0.99

Data are means of duplicate samples.

The optimum Mg^{++} and K^{+} concentrations determined for protein synthesis with SBMV RNA as the messenger were 2.5 mM and 88 mM, respectively. Preliminary studies indicate the optimum SBMV RNA concentration was near 20 ug/100 ul reaction mixture. At lower concentrations of SBMV RNA, activity was not stimulated either by heating the RNA for 3 min at 60° C followed by quick chilling or by the presence of 0.06 uM or 0.12 uM spermine, 0.5 ug/ml actinomycin D, or 10 ug/ml cordycepin [15]. Comparison of SBMV RNA activity in the wheat germ system with comparable concentrations of TMV RNA or BMV RNA indicated that SBMV RNA is less than 25% as efficient as the other viral RNAs in directing *in vitro* protein synthesis (Table 2, Exp. 1). Kinetic experiments indicated that SBMV RNA directed incorporation of ^{14}C-leucine into proteins is linear during the first 60 min of the reaction but is much slower than that for BMV RNA (data not shown).

In vitro *translation of bean polyribosomes*

Polyribosomes from leaves at 6 or 7 day P.I. were chosen for translation studies for three reasons: first, changes in the polyribosome concentration were evident at this time; second, minimal concentrations of potentially interfering SBMV virions were present in the polyribosome preparations of infected plants: and third, a maximal yield of polyribosomes was desired. Equal amounts of polyribosomes from healthy (HP) and SBMV-infected (IP) plants were translated in conditions which were optimal for translation of SBMV RNA in the wheat germ system. Activity could not be enhanced further by the presence of spermine in the reaction mixture.

When polyribosomes from bean leaves 6 day P.I. were translated in a S 23 wheat germ system containing wheat ribosomes, IP polyribosomes stimulated more ^{14}C-leucine incorporation than HP polyribosomes (Table 2, Exp 2).

Table 2

AMINO ACID INCORPORATION ACTIVITY IN WHEAT GERM S 23 EXTRACTS DIRECTED BY VARIOUS RNAS AND BEAN POLYRIBOSOMES

Polyribosomes added	mRNA used	TCA precipitable CPM	Stimulation above background
Experiment I			
None	None	600	0
None	SBMV (6 ug)	11,446	19.1
None	BMV (6 ug)	50,124	83.5
None	TMV (6 ug)	114,470	190.6
Experiment II			
None	None	227	0
None	SBMV (16 ug)	16,962	74.7
6 day P.I. HP_a	None	6,880	30.3
6 day P.I. IP_b	None	8,663	38.2
6 day P.I. HP_a	SBMV (15 ug)	8,278	36.5
6 day P.I. IP_b	SBMV (15 ug)	9,123	40.2

Protein synthesis was measured as L-[^{14}C]-leucine incorporation into trichloroacetic acid (TCA) precipitable material. Incubations contained 15 ul of S 23 wheat germ extract in a standard assay mixture.

[a] Polyribosomes isolated from healthy plants; A_{260}=0.5.

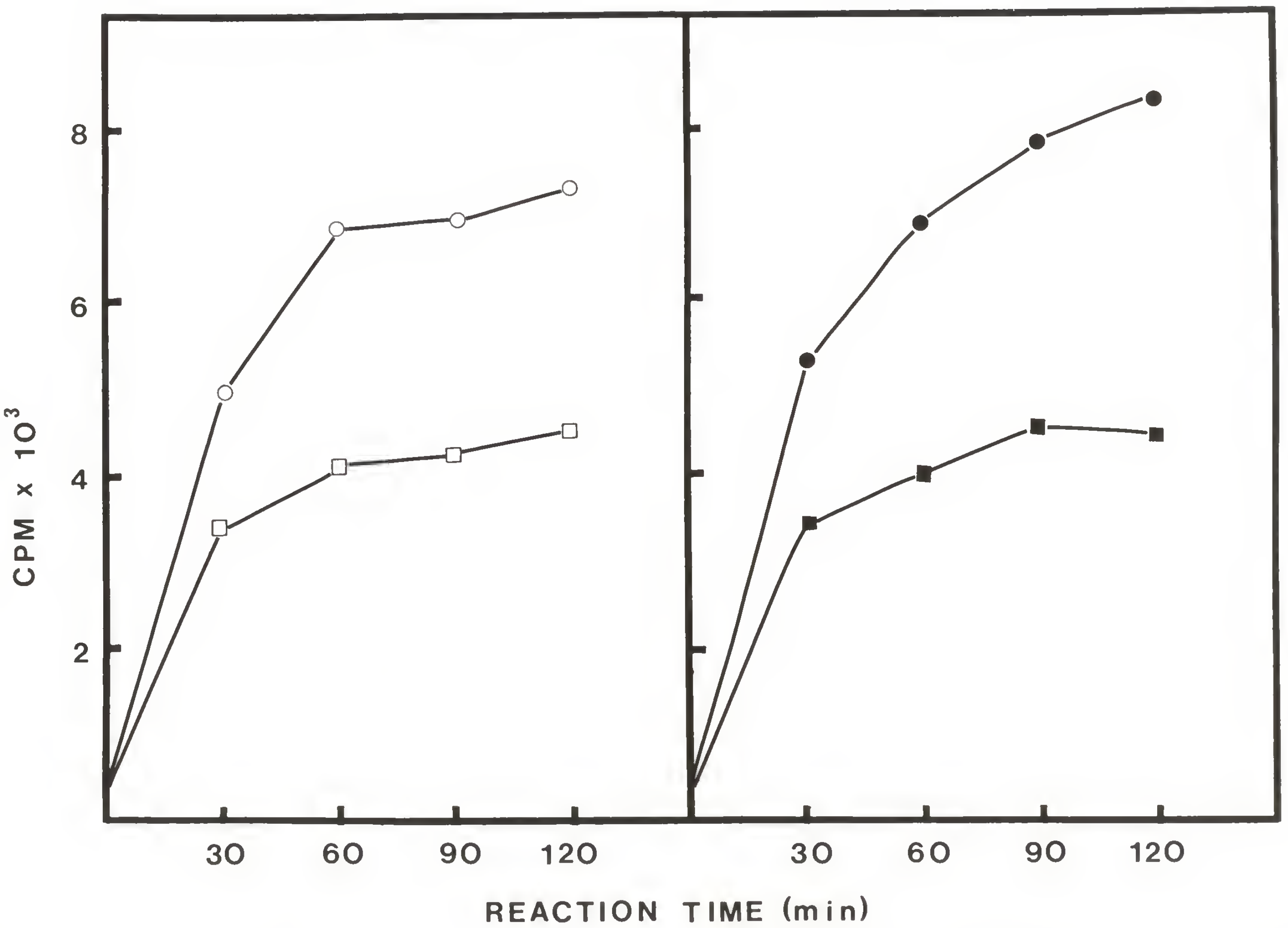
[b] Polyribosomes isolated from SBMV-infected plants; A_{260}=0.5.

Translation reactions treated with aurintricarboxylic acid (ATA), an initiation inhibitor [19,35], were inhibited 40% and 53%, respectively, for HP and IP 6 day P.I. polyribosomes (Fig. 3) indicating a considerable amount of ribosome-bound mRNA was reinitiating protein synthesis on new ribosomes. It also suggest that the ribosome-bound mRNA in the IP polyribosome reactions is approximately 20% more active in reinitiating protein synthesis than the mRNA in the HP polyribosome reactions. *In vitro* activity of HP and IP polyribosomes could be stimulated further by the addition of SBMV RNA to the reaction mixture, and the activity of the IP polyribosomes still remained higher (Table 2, Exp. 2). The additional stimulation occurring with SBMV RNA added to the reactions was probably due to translation of the added mRNA by wheat germ ribosomes present in the system. However, if reaction mixtures containing wheat ribosomes and SBMV RNA are compared with similar reation mixtures with added bean polyribosomes, it is evident that only a small portion of SBMV RNA is translated by wheat ribosomes in the presence of bean polyribosomes. This would suggest that the bean polyribosomes somehow prevent translation of SBMV RNA by the wheat germ ribosomes in the S 23 system.

HP and IP polyribosomes from 7 day P.I. leaves behaved similarly to the 6 day P.I. polyribosomes except that the difference in activity became more pronounced with up to a 2-fold stimulation of the IP polyribosomes over HP polyribosomes (data not shown).

To avoid reinitiation and translation of polyribosomal mRNA by wheat germ ribosomes, a wheat germ supernatant extract lacking wheat ribosomes (S 100) was used in subsequent protein synthesis experiments. Translational activity of polyribosomes from 6 and 7 day P.I. bean leaves indicated differences similar to what was observed using S 23 wheat germ extracts (Table 3).

Figure 3. Time course of *in vitro* amino acid incorporation by polyribosomes isolated from 6 day P.I. healthy (open symbols) and 6 day P.I. SBMV-infected bean plants (closed symbols). Incubation was done in the absence (circles) and in the presence (squares) of 4×10^{-5} M aurintricarboxylic acid.
Incubations contained 0.5 A_{260} units of polyribosomes and 15 ul of S 23 in a standard assay mixture.



CPM x 10³
8
6
4
2
30
60
90
120
30
60
90
120
REACTION TIME (min)

Table 3. Protein synthesis was measured as L-[^{14}C]-leucine incorporation into trichloroacetic acid (TCA) precipitable material. Incubations contained 15 ul of S 100 wheat germ supernatant in a standard assay mixture. Aurintricarboxylic acid (ATA), when added, was 4 x 10^{-5} M.

[a] Polyribosomes isolated from healthy primary leaves of bean.

[b] Polyribosomes isolated from SBMV-infected primary leaves of bean.

[c] 80 S ribosomes pelleted from the wheat germ S 100 fraction.

[d] L-[^{14}C]-leucine incorporation directed by TMV RNA is greater in a S 23 system (Table 2) due to higher concentrations of wheat ribosomes endogenous to the S 23 system.

[e] Addition of ATA to all other S 100 experiments performed resulted in a small decrease in the activity of IP polyribosomes. This experiment is the exception.

Table 3

AMINO ACID INCORPORATION ACTIVITY IN WHEAT GERM S 100 SUPERNATANT DIRECTED BY POLYRIBOSOMES AND SBMV RNA

Polyribosomes added	mRNA added	ATA	TCA precipitable CPM	Stimulation above background
Experiment I				
None	SBMV (10 ug)	-	303	0
6 day P.I. HP (0.7 A)[a]	None	-	3,532	11.6
6 day P.I. IP (0.7 A)[b]	None	-	4,623	15.2
6 day P.I. HP (0.7 A)[a]	None	+	3,494	11.5
6 day P.I. IP (0.7 A)[b]	None	+	3,418	11.3
6 day P.I. HP (0.7 A)[a]	SBMV (10 ug)	-	4,325	14.6
6 day P.I. IP (0.7 A)[b]	SBMV (10 ug)	-	4,989	16.5
Experiment II				
None	None	-	268	0
None	SBMV (8 ug)	-	200	--
WG ribosome (1.0 A)[c,d]	TMV (6 ug)	-	3,259	5.7
WG ribosome (1.0 A)[c]	TMV (6 ug)	+	500	1.9
7 day P.I. HP (1.2 A)[a]	None	-	2,757	10.3
7 day P.I. IP (1.2 A)[b]	None	-	8,058	30.1
7 day P.I. HP (1.2 A)[a]	SBMV (8 ug)	-	2,769	10.3
7 day P.I. IP (1.2 A)[b]	SBMV (8 ug)	-	8,697	32.4
7 day P.I. HP (1.2 A)[a]	None	+	2,553	9.5
7 day P.I. IP (1.2 A)[b]	None	+	8,902[e]	33.2
7 day P.I. HP (1.2 A)[a]	SBMV (8 ug)	+	2,554	9.5
7 day P.I. IP (1.2 A)[b]	SBMV (8 ug)	+	8,666	32.3

However, the addition of ATA to the reaction mixtures had little effect on the activity of HP or IP polyribosomes. This would suggest that little to no reinitiaion of ribosome-bound mRNA was occurring in the reaction, but this does not account for why the IP polyribosomes displayed greater activity than HP polyribosomes if part of the mRNA population in the reaction is not being recycled.

SBMV RNA was added to the reactions (Table 3) to determine if the IP polyribosomes were more efficient in initiating new protein synthesis than HP polyribosomes. Only minor increases could be detected in some experiments but none in others.

The addition of 6-8 ug of purified SBMV virions, free of RNAse activity, to the protein synthesis reactions containing polyribosomes had no effect on the activity of HP or IP polyribosomes (data not shown). This indicates that the small amount of SBMV virions present in IP polyribosomes was not involved in the increased activity of IP polyribosomes.

In vitro *activity of bean monoribosomes*

Attempts were made to determine if the increased protein synthesis activity of IP polyribosomes was related to the ability of bean ribosomes from SBMV-infected plants to more efficiently translate certain mRNAs than ribosomes from healthy plants.

Monoribosomes isolated from bean leaves by standard polyribosome extraction methods following N_2 gas dissociation *in vivo* [16] did not stimulate incorporation of ^{14}C-leucine above background in a wheat germ S 100 system with added SBMV RNA. The addition of S 100 supernatant extracts from etiolated hypocotyls or young leaves of bean to these ribosomes in the wheat germ S 100 reaction also had no effect on the ribosome activity.

Monoribosomes isolated from bean polyribosomes incubated with microccal nuclease (2 ug/11 mg of polyribosomes at 30° C for 45 min) displayed *in vitro* incorporation activity similar to N_2 dissociated ribosomes. Incubation of bean S 100 extracts or N_2 dissociated bean ribosomes with ^{14}C-BMV RNA for 30 min at 30° C indicated that RNAse activity was not present in the ribosomes or bean S 100 preparations. This shows that the lack of protein synthesis activity was not due to the degradation of the added mRNA by endogenous RNAses.

Bean monoribosomes active in ^{14}C-leucine incorporation in a wheat germ S 100 supernatant system were obtained by translating bean polyribosomes to termination in a wheat germ S 100 reaction, isolating the ribosomes, and reintroducing the ribosomes into a fresh wheat germ S 100 reaction for activity assays. Sucrose density gradient analysis of the ribosomes indicated 90% of the A_{254} absorbing material was subunits and monoribosomes with the remaining 10% as 2 and 3-mer polyribosomes. A high background activity of the monoribosome preparation in the S 100 system confirmed the presence of a small amount of ribosome-bound mRNA (Table 4). The protein synthesis activity of both the HP and IP ribosomes was stimulated by adding TMV or SBMV RNA although the stimulation of the latter was minimal. IP ribosomes displayed approximately a 20% increase in TMV RNA directed activity over the activity of HP ribosomes. However, the addition of 0.06 uM spermine nullified this relative increase by stimulating a higher background activity in IP ribosome reactions as well as stimulating activity in reactions containing added TMV RNA (Table 4, Exp. 2).

In a preliminary experiment, the addition of ATA to the reaction mixtures containing bean ribosomes and TMV RNA did not significantly decrease the activity below that of reactions mixtures without ATA. This indicates

Table 4

AMINO ACID INCORPORATION ACTIVITY OF BEAN MONORIBOSOMES OBTAINED FROM BEAN POLYRIBOSOMES

Ribosomes added	mRNA used	ATA	TCA precipitable CPM	Stimulation above background
Experiment I_d				
None	None	-	335	0
WG ribosome $(2.0\ A)_a$	TMV (9 ug)	-	11,000	32.8
7 day P.I. HP $(1.3\ A)_b$	None	-	1,510	0
7 day P.I. IP $(1.3\ A)_c$	None	-	1,550	0
7 day P.I. HP $(1.3\ A)_b$	SBMV (15 ug)	-	1,599	1.0
7 day P.I. IP $(1.3\ A)_c$	SBMV (15 ug)	-	1,773	1.1
7 day P.I. HP $(1.3\ A)_b$	TMV (9 ug)	-	3,950	2.6
7 day P.I. IP $(1.3\ A)_c$	TMV (9 ug)	-	4,946	3.2
Experiment II_d				
7 day P.I. HP $(2.0\ A)_b$	None	-	921	0
7 day P.I. IP $(2.0\ A)_c$	None	-	963	0
7 day P.I. HP $(2.0\ A)_b$	TMV (7 ug)	-	1,206	1.3
7 day P.I. IP $(2.0\ A)_c$	TMV (7 ug)	-	1,509	1.6
Experiment II_e				
7 day P.I. HP $(2.0\ A)_b$	None	-	800	0
7 day P.I. IP $(2.0\ A)_c$	None	-	1,179	0
7 day P.I. HP $(2.0\ A)_b$	TMV (7 ug)	-	1,376	1.7
7 day P.I. IP $(2.0\ A)_c$	TMV (7 ug)	-	1,767	1.4
None	None	-	164	---
None	TMV (7 ug)	-	300	---
WG ribosome $(1.0\ A)_a$	TMV (7 ug)	-	2,704	---

Table 4
(continued)

Ribosomes added	mRNA used	ATA	CPM	Stimulation
Experiment II_e				
7 day P.I. HP (2.0 A)$_b$	None	+	813	0
7 day P.I. IP (2.0 A)$_c$	None	+	1,019	0
7 day P.I. HP (2.0 A)$_b$	TMV (7 ug)	+	1,293	1.6
7 day P.I. IP (2.0 A)$_c$	TMV (7 ug)	+	1,654	1.6
Experiment III_d				
WG ribosome (1.0 A)$_a$	None	-	300	0
WG ribosome (1.0 A)$_a$	TMV (7 ug)	-	3,830	12.7
WG ribosome (1.0 A)$_a$	TMV (7 ug)	+	500	1.6

Protein synthesis was measured as L-[^{14}C]-leucine incorporation into trichloroacetic acid (TCA) precipitable material. Incubations contained 15 ul of S 100 wheat germ supernatant in a standard assay mixture. Aurintricarboxylic acid (ATA), when added, was 4×10^{-5} M. Spermine, when added, was 0.06 uM. Variation between duplicate reactions was less than 150 cpm. Values are means of duplicate samples.

[a] 80 S ribosomes pelleted from the wheat germ S 100 fraction.

[b] Ribosomes isolated from bean polyribosomes of healthy primary leaves.

[c] Ribosomes isolated from bean polyribosomes of SBMV-infected primary leaves.

[d] Reaction mixtures did not contain spermine.

[e] Reaction mixtures contain 0.06 uM spermine.

that ATA was not effective in inhibiting initiation of TMV RNA with bean ribosomes. It also suggests that ATA may not have been preventing the reinitiation of ribosome-bound mRNA in the previous bean polyribosome experiments in the S 100 system.

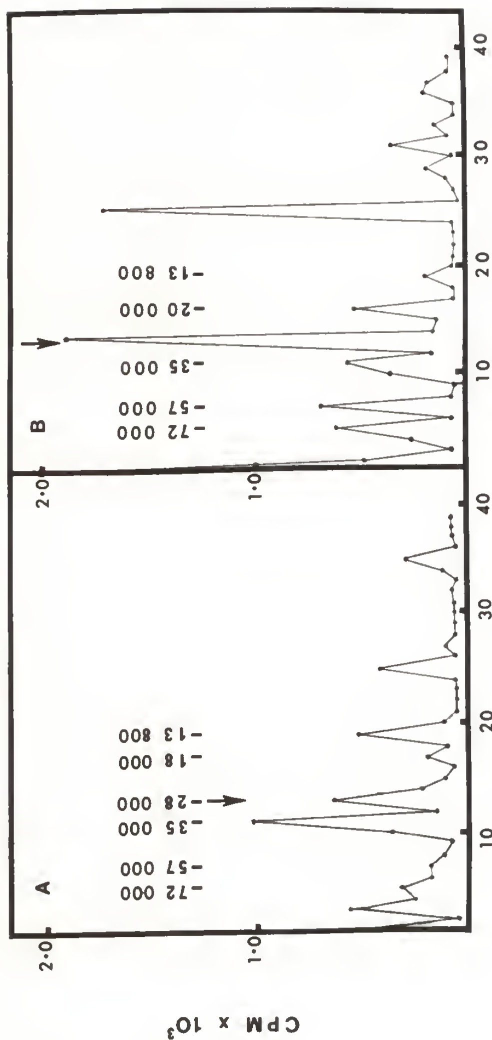
Attempts to determine if the increased translation activity of IP ribosomes and polyribosomes was related to soluble protein factors associated with the ribosomes were unsucessful. Bean ribosomes washed with a 0.5 M KCl buffer [17] prior to translation studies were inactive in the S 100 reaction with added TMV RNA. The washed ribosomes may have needed factors other than those supplied by the wheat germ S 100 to be functional.

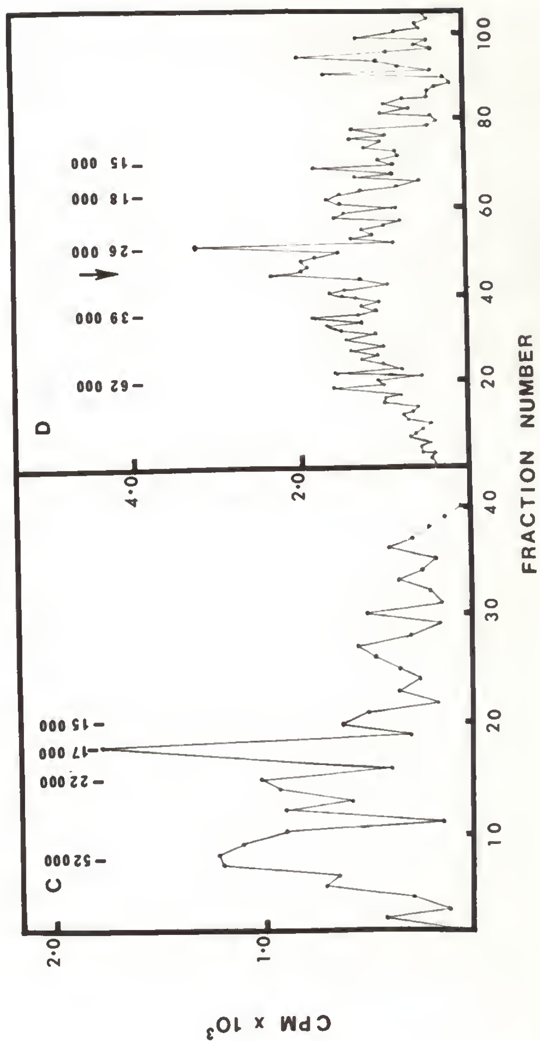
SDS polyacrylamide gel analysis of in vitro *labeled proteins*

Polyribosomes translated in S 23 wheat germ extracts produced a more dispersed profile of labeled polypeptides than when translated in a S 100 extract. This was probably a result of the partial translation of ribosome-bound mRNA by the wheat germ ribosomes. Gel patterns indicated that several similar proteins were synthesized by both HP and IP polyribosomes from 7 day P.I. leaves (Fig. 4). Labeled polypeptides with molecular weights estimated at 14 000, 20 000, 28 000, and 35 000 were found to occur in the gels of all experiments. Other polypeptides with heavier molecular weights occurred but could not be characterized consistently in gels from different experiments. In all experiments, the total amount of ^{14}C-labeled polypeptides was at least 40% higher in reactions from IP polyribosomes than from HP polyribosomes. Analysis of *in vitro* reaction mixtures containing HP or IP ribosomes and TMV RNA indicated that the bean ribosomes were synthesizing polypeptides.

Translation of SBMV RNA in a S 100 wheat germ system yielded a hetero-

Fig. 4. Polyacrylamide gel electrophoresis of ^{14}C-leucine labeled polypeptides synthesized in the wheat germ S 100 system stimulated by: (a) HP polyribosomes; (b) IP polyribosomes; (c) HP ribosomes + TMV RNA; and (d) SBMV RNA translated in a S 23 wheat germ extract. The numbers refer to the molecular weights as derived from standard protein samples following electrophoresis in accompanying gels. The arrow designates the relative position of SBMV coat protein in the gels.


A
B
72 000
57 000
35 000
28 000
18 000
13 800
20 000
2·0
1·0
10
20
30
40
FRACTION NUMBER
CPM x 10³


C
D
52 000
22 000
17 000
15 000
62 000
39 000
26 000
18 000
15 000
2·0
1·0
4·0
10
20
30
40
60
80
100
FRACTION NUMBER
CPM x 10³

geneous group of labeled products occuring throughout the gels. All experiments indicated that no single major product such as the 29 000 M.W. coat protein was being preferentially translated from SBMV RNA.

DISCUSSION

The results reported here show that SBMV infection of bean causes only minor changes in the cytoplasmic polyribosome content. The levels of ribosomes from extracts of diseased plants were only 20 to 30% higher than levels from healthy plants during the time that total ribosome levels in healthy plants were starting to decline (Fig. 2). These results are supported by the observations on the relative levels of ribosomes found in healthy and white clover mosaic virus infected Bataaf beans [34]. Total amounts of rRNA from primary leaves during this time also show concurrent increases in extracts from infected leaves (Table 1). In primary leaves, these small increases in total ribosomes occurred at a time when SBMV was rapidly accumulating in the leaves (Fig. 2). In earlier investigations, Kuhn and Dawson [14] demonstrated that the cowpea strain of SBMV is at its maximum level of synthesis at 4-12 days P.I. in the inoculated primary leaves of cowpeas. This is probably similar in bean. The lag period after ten days in the virus growth curve (Fig. 2) may be a result of translocation of the virions to other parts of the plant [26] such as the trifoliolate leaves.

Minor differences in size distribution of polyribosomes were found concurrently with the change in ribosome content. Extracts from diseased plants displayed a higher proportion of ribosomes as 80 S ribosomes and small polyribosomes (2-4 mer) as indicated by the decrease in the polyribosome to monoribosome ratio. This observation could suggest that the SBMV infection may increase RNAse levels in the cytoplasm of diseased plants which may decrease the effectiveness of the polyribosomal extraction procedures. Alternatively, infection could cause a shift of the polyribosome population from heavy to light polyribosomes and monoribosomes.

The former situation should have been minimized by the use of an extraction buffer with a high pH and ionic strength [12]. The latter situation would suggest reduced initiation of the ribosomes on mRNA or an enhanced progression rate of the ribosomes along the mRNA [33]. It may also suggest that increased levels of shorter ribosome-bound mRNAs are being translated in infected leaves.

Infectivity assays of polyribosome fractions failed to show conclusively whether SBMV RNA was associated in a polyribosome complex or not. Only after the polyribosomes are completely separated from SBMV virions, aggregates, and subviral particles [5] can studies be conducted to determine if SBMV RNA is present in the polyribosomes. Antisera specific for nascent SBMV coat protein subunits could be used to precipitate out SBMV specific polyribosomes. These polyribosomes could then be translated *in vitro* and the radioactive labeled polypeptides could be characterized.

Translation of either 6 or 7 day P.I. polyribosomes from diseased plants resulted in greater activity in the incorporation of amino acids into protein than did polyribosomes of healthy plants. The increased activity of the IP polyribosomes was evident in S 23 wheat germ systems and S 100 wheat germ systems (Tables 2 & 3). The addition of ATA to polyribosomes in the S 23 system suggested that 40-50% of the activity was due to reinitiation of the ribosome-bound mRNA on ribosomes of either wheat germ or bean (Fig. 3). As both HP and IP polyribosomes were inhibited to similar levels with ATA, the increase in activity of the IP polyribosomes without ATA suggests that mRNA from IP polyribosomes may be more efficient in protein sysnthesis in a wheat germ S 23 system. By contrast, the addition of ATA to polyribosomes in a S 100 wheat germ system lacking wheat ribosomes suggests that little to no reinitiation of the ribosome-

bound mRNA was occurring on the bean ribosomes (Table 3). However, bean monoribosome experiments indicated that ATA did not effectively inhibit initiation of bean ribosomes on TMV RNA (Table 4).

Bean monoribosome experiments were performed to determine if the increased activity of IP polyribosomes was associated with increased translation efficiency of the bean ribosomes and their associated protein factors. Ribosomes isolated from bean following an *in vivo* N_2 gas dissociation treatment were non-functional in a S 100 wheat germ system. This treatment has been shown to remove peptidyl-tRNA from the ribosome by preventing the initiation step of protein synthesis [17,31]. Wells and Beever [37,38] have shown that protein factors from ribosomes of pea cotyledons are involved in binding of peptidyl-tRNAs to mRNA to form a ternary complex which binds to the ribosome. A similar complex has been found in wheat germ [32]. The production of runoff bean ribosomes may have resulted in the loss of these types of protein factors, thus rendering the ribosomes non-functional if the wheat germ system's protein factors were incompatible with the bean ribosomes.

Attempts to obtain active bean ribosomes by conversion of the polyribosomes to monoribosomes using micrococcal nuclease were also unsucessful. These ribosomes should still be intact with the necessary protein factors as the nuclease cleaves only the mRNA between each ribosome. Allen and Zamecnik [2] presented evidence which suggests that retoculocyte ribosomes are inactivated by ribonuclease T_1 at the ribosomal binding site for mRNA attachment. A similar condition may have occurred with the nuclease treated bean ribosomes.

Bean monoribosomes functionally active in the S 100 system were obtained by translating polyribosomes *in vitro* to completion. Ribosomes from infected plants displayed higher levels of incorporation with added

mRNA then did ribosomes from healthy plants under similar conditions (Table 4). The addition of spermine to the reaction reduced the relative stimulation of IP ribosomes by increasing the background activity for the IP ribosome reactions. The addition of polyamines such as spermine and spermidine to *in vitro* protein synthesizing systems enhance the activity of viral mRNA translation [29] and cause changes in the Mg^{++} and K^{+} ion requirements for the wheat germ system [3]. Adding spermine to the bean ribosome reaction mixtures may have in some way enhanced the activity of a factor more in the IP reactions than in the HP reactions. Whether the differences in background activity and mRNA stimulation of the HP and IP ribosomes reflects true differences in the functional efficiency of HP and IP ribosomes is not known.

Additional studies are needed on the translational activity of ribosomes and soluble protein factors isolated from healthy and SBMV-infected beans to determine whether modifications are present in the diseased plants and are advantageous to the replication of the virus.

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APPENDIX 1

CHANGES IN RIBOSOME CONTENT DURING INFECTION
(Relative A_{260}/ml/g fresh wt)

Days P.I.	Healthy Primary Leaves			Infected Primary Leaves		
	Polyribosomes	Monoribosomes	Ratio P/M	Polyribosomes	Monoribosomes	Ratio P/M
1	0.77	0.17	4.53	0.77	0.21	3.67
3	2.65	0.26	10.19	2.32	0.22	10.55
4	2.11	0.20	10.55	2.37	0.21	11.29
6	0.98	0.40	2.45	1.27	0.51	2.49
7	1.09	0.24	4.54	1.05	0.34	3.09
9	0.30	0.06	5.00	0.41	0.18	2.28
10	0.12	0.09	1.33	0.12	0.12	1.00
14	0.22	0.17	1.29	0.19	0.20	0.95
17	0.11	0.09	1.22	0.14	0.12	1.17
21	0.19	0.10	1.90	0.12	0.10	1.20
	Healthy Trifoliolate Leaves			**Infected Trifoliolate Leaves**		
	Polyribosomes	Monoribosomes	Ratio P/M	Polyribosomes	Monoribosomes	Ratio P/M
7	0.37	0.23	1.61	0.39	0.29	1.34
9	0.87	0.28	3.11	0.54	0.37	1.46
10	1.14	0.32	3.56	0.44	0.54	0.81
11	0.78	0.37	2.11	0.80	0.43	1.86
12	0.70	0.25	2.80	0.67	0.48	1.40
14	0.27	0.09	3.00	0.28	0.16	1.75
17	0.25	0.10	2.50	0.32	0.16	2.00
21	0.13	0.09	1.44	0.20	0.20	1.00

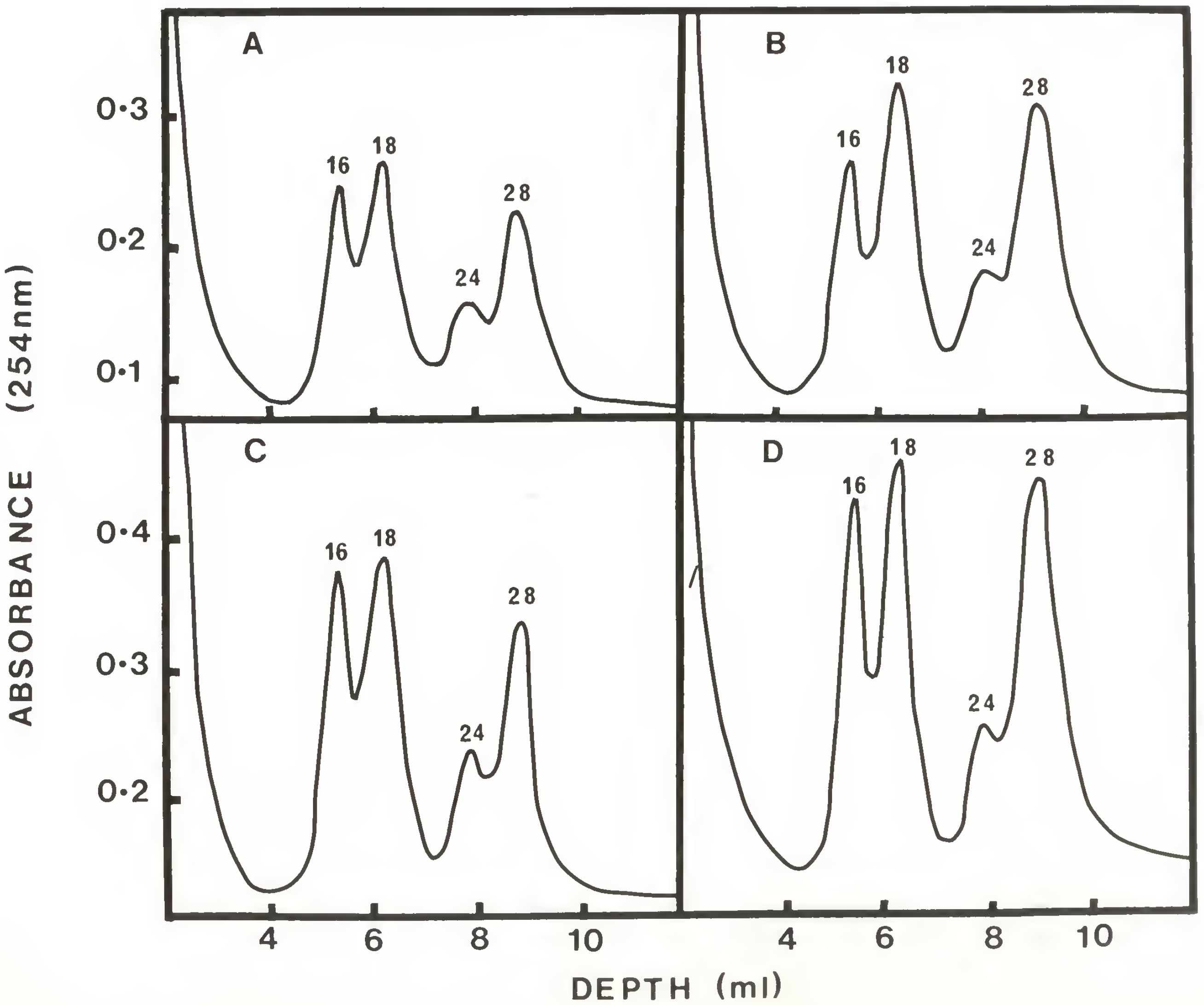
APPENDIX 2

Concentration of buffer-soluble protein in healthy and SBMV-infected primary leaves of Contender bean. Data are means of duplicate samples.

Days P.I.	Healthy (mg/g fresh wt)	Infected (mg/g fresh wt)	Ratio I/H
2	49.0	45.4	0.93
4	237.6	248.0	1.04
6	20.0	40.0	2.00
8	31.0	46.0	1.48
10	39.0	46.0	1.18
12	36.0	42.6	1.18
15	31.0	59.0	1.90
17	24.0	26.0	1.08

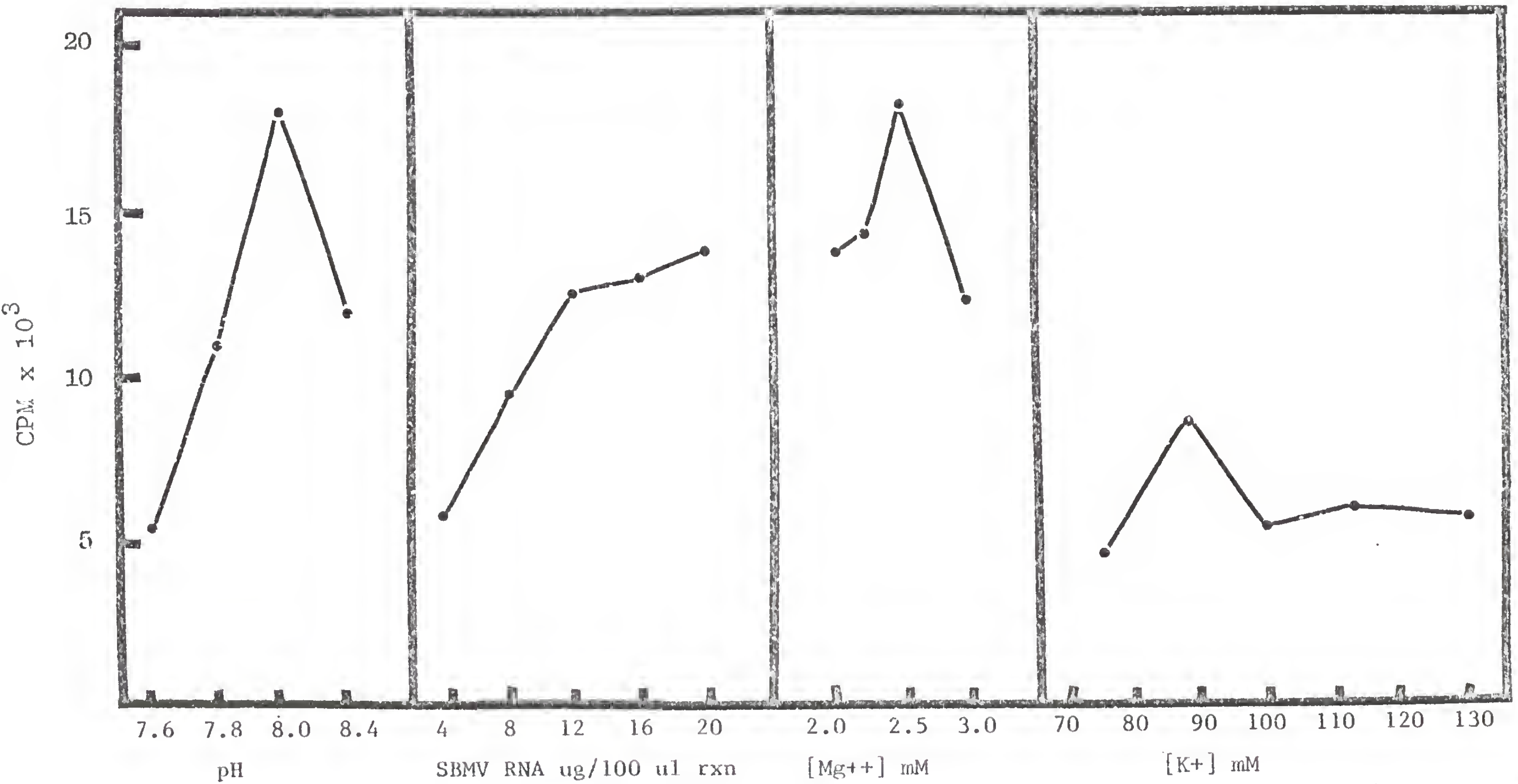
APPENDIX 3

GPS extracts of rRNA from primary leaves of Contender bean after centrifugation on sucrose density gradients as described in MATERIALS and METHODS. RNAs were isolated from (a) 6 day P.I. healthy; (b) 6 day P.I. SBMV-infected; (c) 8 day P.I. healthy; and (d) 8 day P.I. SBMV-infected leaves. Numbers indicate sedimentation positions (S value) of the rRNAs.

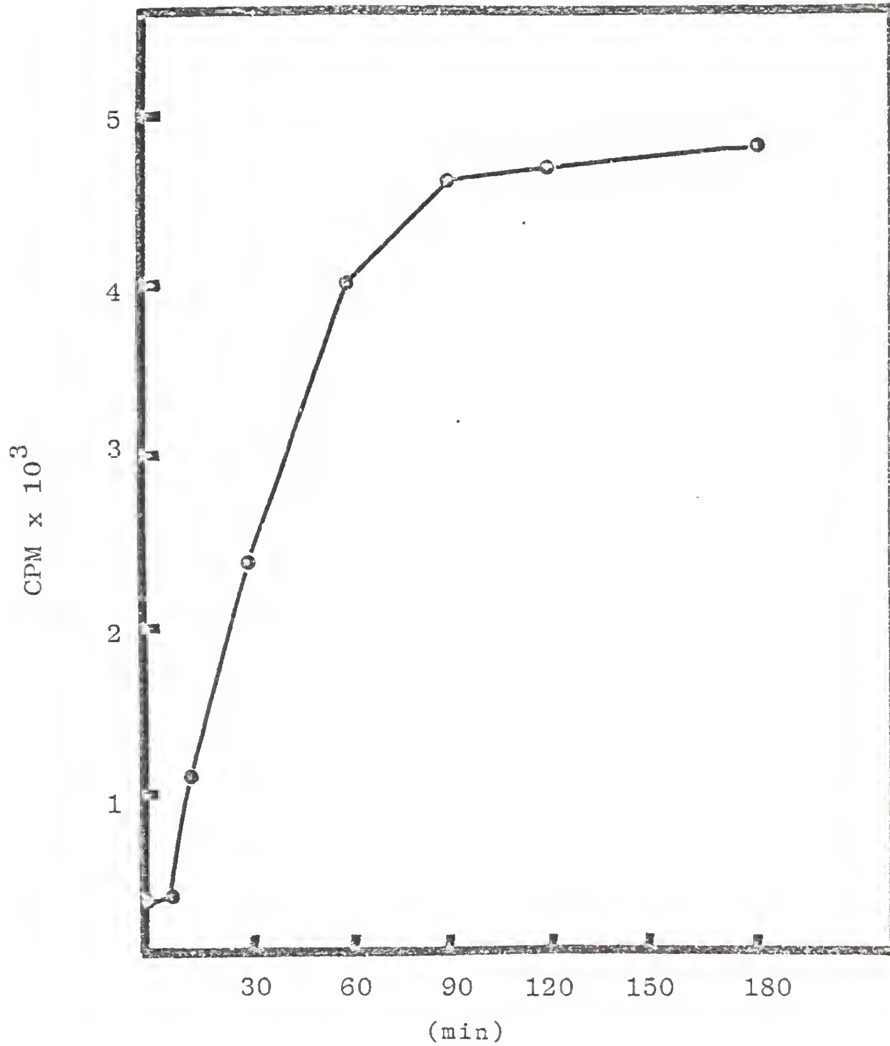

ABSORBANCE (254nm)
0·3
0·2
0·1
0·4
0·3
0·2
A
B
C
D
16
18
24
28
4
6
8
10
DEPTH (ml)

APPENDIX 4

Optimal conditions for *in vitro* protein synthesis in wheat germ S 23 extracts directed by SBMV RNA. 6 ug of SBMV RNA/100 ul reaction was used to determine pH and cation optimums. Data are means of duplicate samples.



APPENDIX 5



Kinetics experiment of SBMV RNA directed radio-activity incorporation. 4 ug of SBMV RNA was incubated for various lengths of time at 23° C in a standard S 23 wheat germ reaction mixture.

QUANTITATIVE AND QUALITATIVE COMPARISONS OF POLYRIBOSOMES FROM HEALTHY AND SOUTHERN BEAN MOSAIC VIRUS-INFECTED CONTENDER BEAN

by

JOHN FRANCIS RAJEWSKI

B. S., Fort Hays State University, 1975

AN ABSTRACT OF A MASTER'S THESIS

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requirements for the degree

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Manhattan, Kansas

1978

ABSTRACT

The protein synthesizing characteristics of cytoplasmic polyribosomes from leaves of *Phaseolus vulgaris* L. cv. Contender infected with southern bean mosaic virus (SBMV) were studied. Only minor changes were observed in total ribosomes from the inoculated primary leaves and the first systemically infected trifoliolate leaves before symptoms appeared and virus accumulated. Preparations from infected plants contained fewer polyribosomes, but the quantities of ribosomes were equal in healthy and infected plants.

Polyribosomes from primary leaves of healthy and infected plants were translated in a wheat germ *in vitro* protein synthesizing system. Polyribosomes from infected leaves 6-7 days post-inoculation were more efficient in completing nascent polypeptide chains than were polyribosomes from comparable healthy leaves. The polypeptides labeled *in vitro* were characterized on SDS-polyacrylamide gels.

80 S ribosomes from healthy and infected bean were tested for their ability to initiate protein synthesis in a wheat germ supernatant system under the direction of added viral mRNA. Ribosomes from infected plants were about 20% more efficient in directing protein synthesis with tobacco mosaic virus RNA. The addition of 0.06 uM spermine eliminated this difference in stimulated activity.