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STUDIES ON SOYBEAN MOSAIC VIRUS

II. Purification and some properties of the virus

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Introduction

GALVEZ (1963) made first success in partial purification of the soybean mosaic virus (SMV) by using a combination of rate and equilibrium zonal centrifugation. Aggregation and low concentration of the virus are emphasized as the main problem in the purification. Partial purification of SMV was also made by De VASCONCELOS (1964) using WETTER's method (1960), slightly modified, for the preparation of the antiserum. ROSS (1967) reported a procedure for the purification of this virus with higher purity sufficiently to make antiserum free from host components. However, this purification method gave still lower yield of purified virus. It still seems to be the problem in taking advanced study such as biophysical and biochemical one. The improvement of the virus purification responsible for higher yield and homogeneity is worthy to be studied. The present investigation was, thus, made in an attempts to solve this problem. Some of the properties of purified virus are also described here.

Materials and methods

Test plants and virus replication

The source of the virus is the same as described previously (HAN and MURAYAMA, 1970). A mild strain of SMV isolate T-109 was mainly used in this investigation. The virus was obtained from a single lesion isolate which was prepared by cutting a single lesion from local-lesion tissue of an inoculated leaf of bean variety Tsurunashi-kintoki. The virus was inoculated on soybean variety Tokachinagaha which was kindly supplied by Tokachi Agricultural Experiment Station, Hokkaido, Japan. The test plants were

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grown in the pot and kept in the greenhouse at the temperature 25~28°C. Soybean plants were inoculated on both primary leaves when the first trifoliolate leaf was not expanded. Carborundum-dusted leaves were inoculated with a 1:10 dilution in 0.01 M phosphate buffer (pH 7.0) of an extract of young, SMV-infected soybean leaves. Adequate precautions were taken to avoid any possible contamination of the virus during the experiments. Leaves of infected plants were harvested 21 days after inoculation where the primary leaves showed necrotic local lesions and the upper trifoliolate leaves showed systemic mosaic infections.

Bioassay of the virus

Bean variety Tsurunashi-kintoki was used as the assay plant throughout the experiments. Local lesion assays of the virus were made on the primary leaves of young plants to determine the relative infectivity and the concentration of the virus. Usually each sample was inoculated to 8-10 half-leaves. The number of local lesions produced by a series dilutions of the virus sample was counted and fitted to a dilution curve of the virus.

Purification procedure

The virus was purified by a modification of the method described by Ross (1967). All the treatments for purification of the virus were carried out at or near 4°C. Hitachi model 40-P ultracentrifuge was used in this experiment. The infected plants were harvested 21 days after they had been inoculated, frozen and thawed, and then ground in a meat grinder adding a buffer of 0.5 M sodium citrate containing 1% 2-mercaptoethanol adjusted to pH 6.5 with 0.1 N HCl in a proportion of 2 ml of buffer added to each gram of infected leaves. The pulp was extracted by squeezing through two layers of cheesecloth. Eight percent of n-butanol was mixed with the extracted sap. The mixture was then stirred slowly for 10 minutes. The aqueous extract was stored overnight in a cold room (4°C) and the emulsion was broken by low speed centrifugation at 10,000 rpm (8,500 g) for 10 minutes. The resulting supernatant was clear amber in color. It was filtrated through glass wool. The clarified supernatant was centrifuged in RP 30 angle-head rotor at 30,000 rpm (75,000 g) for 90 minutes. The resultant pellets were resuspended in 2 ml of cooled 0.01 M sodium borate buffer (pH 8.3), homogenized in a Teflon glass homogenizer and the suspension was clarified at 10,000 rpm for 10 minutes to remove insoluble and aggregate materials. The clarified suspension was recentrifuged at 30,000 rpm for 90 minutes and pellets were resuspended in borate buffer and clarified as mentioned above. Final suspension, thus prepared, was used as the virus sample for zonal

density-gradient centrifugation (BRÄKKE, 1967). Gradient columns were prepared by layering 4, 7, 7, and 7 ml of 0.01 M borate buffer (pH 8.3) containing 10, 20, 30, and 40 percent (W/V) sucrose, respectively, in BECKMAN cellulose nitrate centrifuge tube (1 × 3). After preparation, the gradient was allowed to make continuous diffusion by overnight in cold room at 4°C before use. Two ml of the virus suspension was floated on the top of each column and centrifuged at 25,000 rpm (63,500 g) for 120 minutes in a RPS 25-A swinging bucket rotor of the Hitachi model 40 P ultracentrifuge at refrigerated temperature. The virus zone was located by illuminating the tube lengthwise with a Nikon illuminator. Zone depths were measured directly outside the tube and the photographs were taken immediately after the tubes were removed from the rotor. The virus zone was removed from the tube at the side by puncture with a gauge needle and syringe. The separated fractions were then diluted by cooled buffer and followed by one cycle of differential centrifugation. Usually the fractions were centrifuged at 40,000 rpm (100,000 g) in a RP 40 rotor for two hours to pellet the virus. This pellet was suspended in deionized distilled water (pH 7.0), saline solution or phosphate buffer solution (pH 7.0) according to the experiments. The virus thus prepared followed by a low speed centrifugation was preserved in refrigerator (-45°C) until use.

Ultraviolet absorption

Ultraviolet absorption spectra of purified virus preparations were determined with a Hitachi model EPV-2A spectrophotometer. Determinations were done in 0.05 M borate buffer (pH 8.3). KALCKAR's equation, protein in mg = $1.45 E_{280} - 0.74 E_{260}$, was used in this investigation.

Antiserum

Antisera used for serological tests were obtained from rabbits injected intramuscularly with the antigen. For injection, one ml virus suspension containing about 0.1 mg protein in saline was emulsified in equal volume of FREUND's complete adjuvant. Each rabbit received four times of 0.1 mg antigen at one week intervals. The titer of the antiserum was determined and the rabbits were bled 14 days after final injection. The sera were preserved by freezing in a refrigerator before use. An antiserum against normal component of healthy soybean plants was also prepared to detect normal antigens in the purified virus preparation. Normal serum was collected prior to immunization. Serological tests were made, either by interface precipitin tests or by Ouchterlony agar double-diffusion tests. The interface precipitin tests were carried out in glass tubes in which serial 2-fold dilution of anti-

serum was made in physiological saline containing 10 percent of glycerine and upper layer with antigen. The tubes were partially immersed in water bath at 37°C incubated for 30 min. Readings were taken at the end of this period. Ouchterlony agar double-diffusion tests were done on microscopic slide. The gel consists of 0.85% (W/V) Bifco Noble agar with 0.85% (W/V) sodium chloride in 0.01 M phosphate buffer (pH 7.0) [with 0.004% (W/V) sodium azide. Two fold dilution of antigen and antiserum were utilized to determine the titer of antiserum. The antiserum prepared from the normal plant antigens had a titer of 1/32.

Electrophoresis

The preparation of purified virus (5mg/ml) suspended in deionized distilled water (pH 6.9) was examined in a Toyo Roshi model 3BA8 electrophoresis apparatus. Separax (Cellulose acetate) produced by Jo Ko Sangyo Co., was used as the supporting membrane. A ultramicro-pipette was used to applying 1.2 μ l virus preparation on a strip of Separax (3 \times 6 cm). The electrophoresis was carried out for 45 min. at room temperature at the current of 20 mA/CM. After electrophoresis, the Separax strips were removed, stained with a water solution containing 6% trichloroacetic acid and 0.8% Ponceau 3R, and destained by repeated washing in 1% acetic acid. Migration distance was determined by measuring the distance between the apex of boundary. Migration rate was expressed as centimeters $\times 10^{-3}$ min⁻¹.

Measurement of sedimentation coefficient

The sedimentation coefficient was determined in 0.1 M NaCl (pH 7.0) at the virus concentrations of 0.5, 0.4, 0.32 and 0.256 mg per ml using a Spinco model E analytical ultracentrifuge. Sedimentation rate measurements were made from the photographs of Schlieren patterns taken at 4-min intervals at bar angle of 60 degrees starting 5 min., after H-A rotor reached 20,410 rpm. The rotor temperature was 20°C. The observed sedimentation value was converted to S_{20}^0 , W.

Results

Purification

Higher yield and homogeneity of the purified virus was obtained by the method as mentioned in this paper. The treatment of the infected juice with eight percent *n*-butanol for clarifying is better than seven percent in removing more plant components during first low speed centrifugation. The primary pellets obtained from first high speed centrifugation is ember colored and somewhat easy to suspending in borate buffer. The infectivity of purified

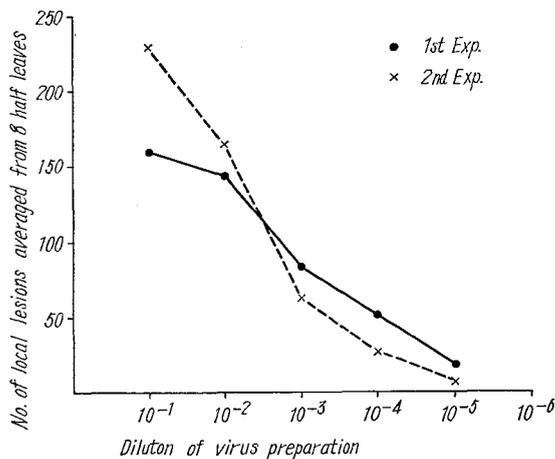


Fig. 1. Bioassay of purified soybean mosaic virus (1 mg/ml) on primary leaves of bean variety, Tsurunashi-kintoki.

TABLE 1. Characters of different fractions from density gradient column.

Exp.	Virus isolate	Fractions ^a	UV absorption		Serological ^b reaction	Infectivity ^c
			Max/Min ratio	280/260 m μ ratio		
1	SMV (T-109)	U	1.14	0.77	+	±
		M	1.27	0.72	+	++
		B	1.11	0.80	+	+
2	SMV (T-109)	U	1.19	0.79	+	±
		M	1.37	0.65	+	++
		B	1.27	0.71	+	+
3	SMV (T-121)	U	1.23	0.76	+	±
		M	1.27	0.68	+	++
		B	1.28	0.67	+	+

a. U, M, and B represents upper, middle and bottom zone, respectively in density gradient column.

b. Reaction with homologous antiserum determined by interface precipitin tests.

c. Bean variety, Tsurunashi-kintoki was used as an indicator; ± ~ ++ represents the degree of infectivity.

virus preparations thus clarified was not found to be decreased in comparison with seven percent butanol treatment. After two cycles of differential ultracentrifugation, the virus produced a bright zone located in 2.6 to 2.7 cm below the meniscus in rate sucrose density gradient tubes. It was not found from

the preparation of healthy soybean leaves similarly prepared. Usually, three zones were visible when observed under vertical beam of light against a dark background. The uppermost zone is about 2 mm in wide, white and somewhat yellow in color located 2.4 cm below the meniscus. The second zone, a sharp light-scattering boundary located at 2.6 cm below the meniscus. The intensity of light scattering in this band, was greatest at 2.6 cm and in most cases gradually became less at lower depth, similar to that described by Ross (1967). A lesser degree of light-scattering was visible at depth below this band. In some cases, particularly the virus concentration is higher enough and this zone is visible. The top zone having a lowest infectivity suggested that it contained short rods or fragments of various size of viral particles. Higher infectivity was found in middle zone, and hence designated as virus zone. The infectivity of bottom zone is lower than middle zone. Therefore, the bottom zone component presumably contained aggregated virus formed before or during purification (PURCIFULL et al., 1968). Fractions from the virus zone were collected and combined. The virus suspension was given a additional cycle of differential centrifugation for removing the sugar and then tests for infectivity and used for further works as purified virus. The concentrated suspension of the virus was colorless, opalescent and highly infectious. Serological tests denote that all the zones gave positive reactions to SMV antiserum in interface precipitin tests. The infectivity of the purified preparation varied with the preparations. One preparation inoculated 24 hours after purification produced 167, 132, 81, 52 and 21 lesions on bean leaves at the concentrations $1.0 \text{ mg} \times 10^{-1}$, $1.0 \text{ mg} \times 10^{-2}$, $1.0 \text{ mg} \times 10^{-3}$, $1.0 \text{ mg} \times 10^{-4}$ and $1.0 \text{ mg} \times 10^{-5}$, respectively. The virus preparations assayed on bean primary leaves had a dilution end points of 10^{-4} to 10^{-6} from one ml virus suspension containing one mg virus. Yields of purified virus were about 1 to 75 mg per kg infected leaves. Purified preparations lost infectivity slowly when stored in refrigerator (-45°C). No apparent changes occurred in infectivity when it was refrigerated for two months. However, the virus appeared to degrade or denatured rapidly especially the virus suspended in saline solution when the preparations were allowed to stand at room temperature for two to three days.

Ultraviolet absorption

The ultraviolet absorption spectrum of purified virus was similar to that reported by Ross (1967). The typical absorption curve of one sample, which gave a single peak in the analytical ultracentrifugation, is shown Fig. 2. It had a maximum absorption occurred at $260 \text{ m}\mu$ and a minimum at $245 \text{ m}\mu$. The max/min ratio was 1.14 to 1.38, and $280/260 \text{ m}\mu$ ratio was 0.64 to 0.74

according to the preparations, indicating about 8.75 to 15.25% nuclear acid content (WARBURG and CHRISTIAN, 1941). On the other hand, the fractions of the virus from density-gradient tubes gave different ultraviolet absorption in RNA content. The results obtained from several virus preparations indicated that RNA content in major zone of density-gradient tube usually gave higher concentration. The fractions collected from above or below major zone showed a lower content of RNA.

Serological Activity

The purified virus is not reacted with the antiserum against plant components. In no case was found that the antiserum against purified virus reacted with healthy plant antigen. The maximum titer of antiserum determined by interface precipitin tests was 1/2048 to 1/4096 according to the preparations. Similar end points were found in tests with homologous and heterologous antigens. In double diffusion tests, always gave a single line of precipitation including the materials collected from different fractions of density-gradient tube.

Electrophoresis

The purified virus gave a single boundary, and had a mobility of $10^{-3} \times 5.5 \text{ cm min}^{-1}$. This result indicates that the purified virus is higher in homogeneity which remained a uniform of electrophoretic pattern. The virus was moving to positive pole when submitted to electrophoresis in Separax, indicating a negative charge on the particle surface.

Analytical ultracentrifugation

A virus solution prepared from infected leaves showed only a single peak examined in the analytical ultracentrifuge. The sedimentation coefficient, determined in 0.1 M NaCl, increased with decreasing virus concentration; and on correction to the standard solvent density and viscosity of water at 20°C gave an extrapolated value of 143 Svedberg units at zero concentration. This result indicated that the purified virus was higher in homogeneity. Virus preparation before density-gradient centrifugation was also examined

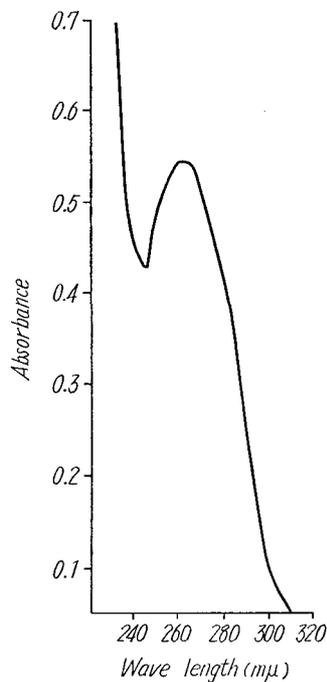


Fig. 2. Ultraviolet absorption spectrum of the soybean mosaic virus. The virus was suspended in 0.01 M borate buffer, pH 8.3.

TABLE 2. Ultraviolet absorption of purified soybean mosaic virus.

Exp.	SMV isolates	Max/Min ratio	280/260 m μ ratio
1	SV-15	1.26	0.67
2	SV-15	1.29	0.66
3	SV-15	1.26	0.69
4	T-1	1.28	0.67
5	T-9	1.38	0.64
6	T-38	1.18	0.73
7	T-38	1.25	0.66
8	T-41	1.30	0.68
9	T-109	1.32	0.67
10	T-109	1.27	0.66
11	T-109	1.23	0.74
12	T-109	1.27	0.72
13	T-109	1.37	0.65
14	T-121	1.25	0.71
15	T-121	1.27	0.66
16	T-121	1.31	0.68
17	T-121	1.14	0.71

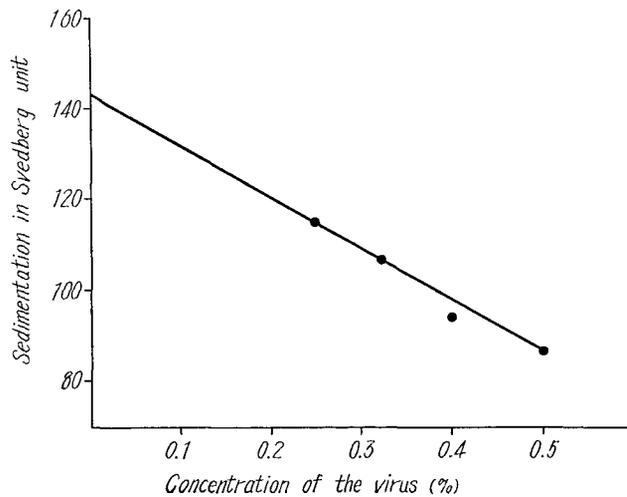


Fig. 3. Plot of the sedimentation coefficients as a function of SMV concentration.

in analytical ultracentrifuge. The Schlieren pattern of analytical ultracentrifuge gave a idea that besides of the main peak there were some fast and slow peaks which probably was caused by aggregates and the fragments of the virus. The result was correlated to the zones forming in the density-gradient column. From this point of view it indicated that the sucrose density-gradient technique has been found to be extremely useful procedure for purifying SMV in higher homogeneity.

Discussion and conclusion

Virus in PVY group is difficult in purification because of side by side and end to end aggregations of the virus during purification (PURCIFULL et al., 1968). Based on the infectivity tests, it indicated that some particles lost during differential centrifugation was approximately caused by virus aggregation. In measurement of the virus particles, it was found that some short fragments existed in the infected juice when observed under the electromicroscopy. The top zone appeared in density-gradient column may be mainly correlated with the short rod of the virus. Result obtained from analytical ultracentrifugation which denoted that sucrose density-gradient centrifugation was emphasized as a useful procedure in removing virus fragments and aggregates. Data given in this paper were clear that the purified virus was higher in homogeneity.

So far as the ultraviolet absorption of the purified virus is concerned, the result is not identical to that reported by ROSS (1967). The maximum absorption was occurred at 260 $m\mu$, but not at 260–265 $m\mu$. On the other hand, RNA content of the virus determined from the ultraviolet absorption was also not similar to that described by ROSS. DAMIRDAGH and SHEPHERD (1969) reported a new method in purification of the virus in PVY group free from the aggregations of the virus particles. Using 2-mercaptoethanol alone is not emphasized as useful in preventing the aggregation of the virus in this group. Satisfaction of the purification was obtained by using this chemical combined with urea. However, the urea was capable of degradate the particles of SMV as denoted from other experiment. Whether the urea is useful or not for the purification of SMV is worthy to be studied. The sedimentation coefficient of SMV, determined by analytical ultracentrifuge, is not similar to tobacco etch virus as reported by PURCIFULL (1966) and other viruses in PVY group (BRANDES and BERCKS, 1969).

Summary

SMV was purified by a modification of the method described by Ross. The purified virus was very high in purity as determined from the serological tests and analytical ultracentrifugation. Virus preparation had an ultra-violet absorption with a maximum at 260 $m\mu$ and minimum at 245 $m\mu$. The maximum/minimum ratio was 1.14 to 1.38, and 280/260 $m\mu$ ratio was 0.64 to 0.74. The Schlieren pattern obtained by analytical ultracentrifugation showed only one peak. The sedimentation coefficient of SMV is about 143S. The electrophoretic pattern of the virus on Separax gave one boundary with a mobility of $10^{-3} \times 5.5 \text{ cm min}^{-1}$.

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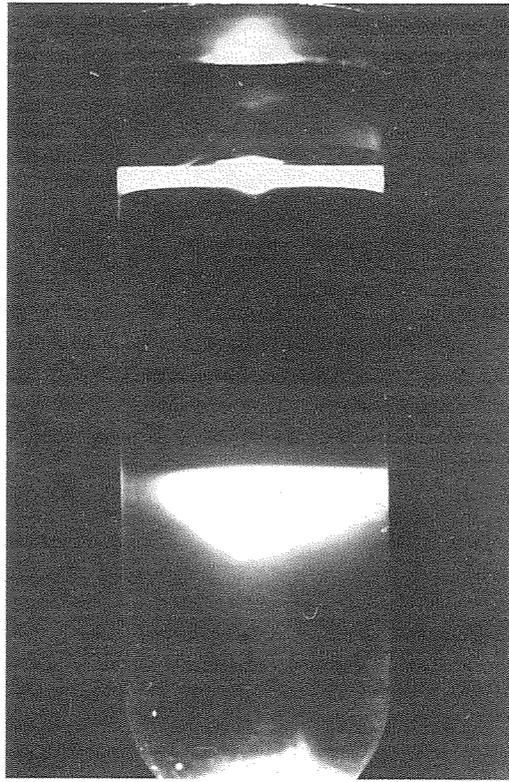
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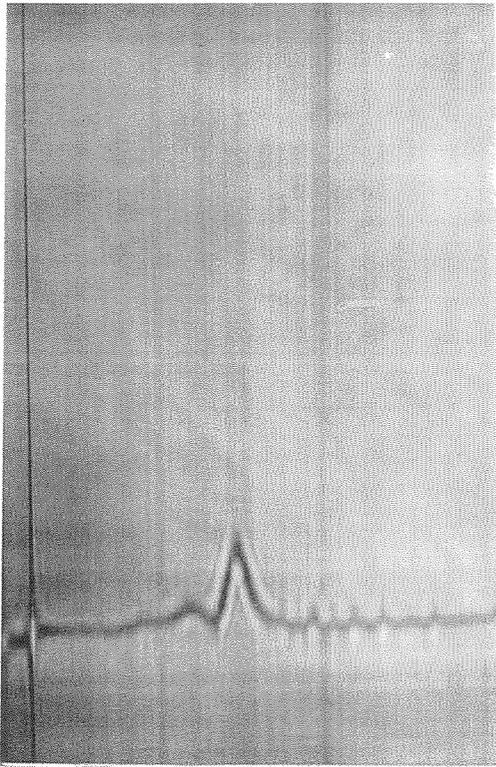
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Explanation of plate

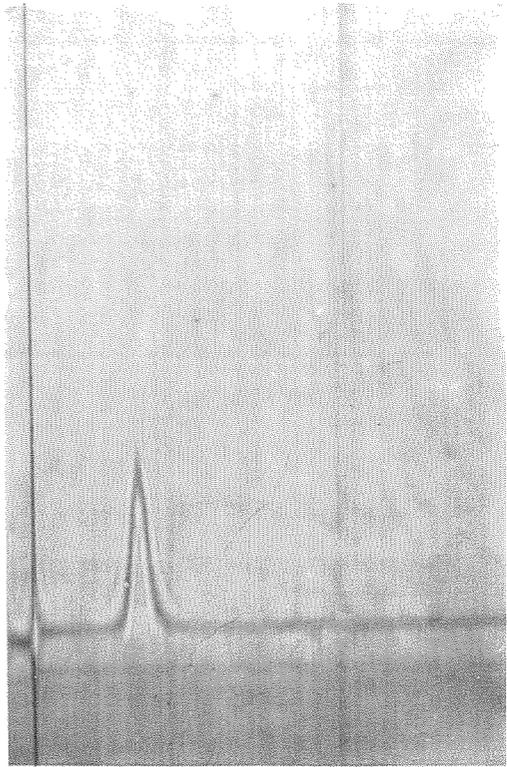
- A. Rate density gradient tube containing soybean mosaic virus after two cycles of differential centrifugation viewed under direct overhead light, showing the virus boundary, the upper zone is not visible in the photograph.
- B. Schlieren pattern of a partial purified soybean mosaic virus, showing several boundaries, photograph was taken 32 minutes after a speed of 20,410 rpm had been reached. Sedimentation was done from left to right.
- C. Schlieren pattern of a purified virus showing a single boundary. Photograph was taken 8 minutes after a speed of 20,410 rpm had been reached. Sedimentation was done from left to right. The virus has a sedimentation coefficient of 143 S.



A



B



C