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STUDIES ON THE RNA OF BACTERIOPHAGE MS2

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Some of the nucleic acid infectivity assays reported here were done with the competent assistance of Mrs. Gloria Davis. This work was supported by training grants from the United States Public Health Service.

ABSTRACT

The first part of this dissertation contains a publication which discusses the purification and properties of bacteriophage MS2 and its RNA, including the RNA content of the virus, and light scattering particle weights and sedimentation properties of the virus and its RNA. A short discussion of this publication in light of later results is included.

An infectivity assay for MS2-RNA is characterized in Part II. The results of varying several parameters of the assay are presented, and the effects on the assay of competing RNA's and external ribonuclease are discussed.

Part III presents the initial kinetics of degradation of MS2-RNA by ribonuclease, heat, and alkali, as followed by the exponential decline in RNA infectivity. The turnover number of pancreatic ribonuclease is independent of RNA concentration between 10^{11} and 10^{15} RNA molecules per ml. Inactivation at high pH is almost proportional to the hydroxyl ion concentration between pH 11 and 12.3. The activation energy for thermal inactivation is 22 kcal./mole.

Part IV contains studies on the denaturation of MS2-RNA, of a double-stranded form of RNA, and of rG:rC by thermal means and with organic solvents. Evidence is presented that in dimethylsulfoxide RNA is completely

denatured at room temperature.

Part V describes the centrifugal properties of MS2-RNA. The RNA is shown to possess no covalent configurational restraints by the presence of but a single component when sedimented under denaturing conditions, by the correspondence between the decline in infectivity produced by ribonuclease and the production of RNA fragments, and the coincident sedimentation of the RNA infectivity with the bulk RNA under a variety of conditions, including sedimentation through dimethylsulfoxide. The RNA chain does hydrogen-bond to itself, however, giving rise to multiple, homogeneous, infective components under certain conditions.

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Part I

PURIFICATION AND PROPERTIES OF BACTERIOPHAGE MS2 AND OF ITS RIBONUCLEIC ACID

Before undertaking an intelligent investigation of the replication or detailed properties of a virus or its nucleic acid, it is necessary to be able to purify large amounts of material and to know certain basic physical and chemical properties. The following publication contains our first observations on MS2 and its RNA, and gives this background information. This article is reproduced from the Journal of Molecular Biology (1963), vol. 7, pp. 43-54, by permission of Academic Press Inc. (London) Ltd.

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Purification and Properties of Bacteriophage MS2 and of its Ribonucleic Acid

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Methods are described for the isolation in pure form of bacteriophage MS2 and of its RNA.

The virus has a particle weight of $3{\cdot}6 \times 10^6$, and from phosphorus content is estimated to be 32% RNA by weight. The particle weight of the isolated RNA is $1{\cdot}05 \pm 0{\cdot}1 \times 10^6$, indicating that there is one molecule of RNA per virus particle.

The RNA is shown to have several unusual properties. In 0·2 M-NaCl it has a very small radius of gyration and a large sedimentation coefficient for its molecular weight, indicating a very compact structure. Upon heating the RNA in 0·14 M NaCl solutions, the ultraviolet absorbancy transition has a midpoint at 76°C, suggesting a high degree of hydrogen bonding. In lower salt concentrations the RNA aggregates; in 0·02 M-NaCl the observed molecular weight is twice that in 0·2 M-NaCl, and in 0·003 M-tris it is 3 times this value.

1. Introduction

Bacteriophage MS2 is a small virus, originally isolated by Dr. Alvin J. Clark, which contains RNA as its nucleic acid (Davis, Strauss & Sinsheimer, 1961; Davis & Sinsheimer, 1963). In order to study the replication of such an RNA virus it is necessary to know the physical and chemical properties of the particle; several of these parameters have been determined on purified virus preparations. In the course of this investigation it became apparent that the RNA of the virus possesses several unusual properties; these have been studied in more detail.

2. Materials and Methods

(a) *Media and assay of the virus*

The media used and the method of assay of the virus are as given in Davis & Sinsheimer (1963), with the exception that bottom agar contains the same ingredients as MS broth plus 1% Bacto-agar.

(b) *Growth of the virus*

For mass cultures, 7 l. of sterile medium are inoculated with *E. coli* C 3000 and the culture aerated vigorously at 37°C. When the bacterial concentration reaches 4×10^8/ml. (as observed with a Petroff-Hausser counter), virus is added to produce a multiplicity of 5. Lysis begins about 2 hr after infection; foaming at lysis is controlled by the addition of Dow-Corning anti-foam B. Aeration is continued for 6 hr after addition of virus. The titer of lysates so produced has varied from 2×10^{12} to 4×10^{13}/ml.

(c) *Purification of the virus*

The method used is a modification of the ϕX174 purification procedure of Sinsheimer (1959*a*).

To the lysate is added 280 g ammonium sulfate/l. with vigorous stirring (in later preparations 25 ml. 0·1 M-EDTA, pH 7, have also been added per liter). After 2 hr in the cold, the precipitate is spun out in the Spinco model K continuous-flow centrifuge. The supernatant solution contains about 10% of the initial virus titer and is discarded.

The precipitate is thoroughly mixed with 70 ml. (for 7 l. of lysate) of a buffer containing 0·1 M-NaCl and 0·05 M-tris (2-amino-2-hydroxymethylpropane-1 : 3-diol), pH 7·6.† To the suspension is then added 70 ml. of Freon-11 ($CFCl_3$, obtained from the Virginia Chemicals and Smelting Company, West Norfolk, Virginia) (Gessler, Bender & Parkinson, 1956). The resulting mixture is shaken by hand for 3 min, and the emulsion then broken by centrifugation for 10 min at 12,000 *g*. The aqueous layer is decanted; it contains approximately 60% of the initial virus titer. To the Freon layer is added 30 ml. of the NaCl–tris buffer, and the mixture treated as before. About 5% of the initial virus titer is recovered in the second aqueous layer.

To the pooled aqueous layers, 0·550 g CsCl/g is added to make the density of the resulting solution 1·38 ± 0·01. The solution is divided into twelve lusteroid tubes and spun in the 40 rotor in the model L centrifuge for 24 hr at 37,000 rev./min at 2°C (Meselson, Stahl & Vinograd, 1957).

Upon completion of the run the rotor is allowed to decelerate without brake. The tubes are then carefully removed. Usually two bands are visible, with the virus band located lower in the tube. The virus band is removed from each tube with a syringe, and the material pooled. The combined material is placed in two or three tubes and centrifuged a second time. Upon completion of the second run the virus band in each tube is collected by punching a small hole in the bottom of the tube and allowing the material to drip out of the bottom. The virus band is easily visible and can be collected in a separate container. The virus isolated from each tube is pooled and dialysed against three changes of 1 l. each of a buffer containing 0·1 M-NaCl and 0·01 M-tris, pH 7·6 (or against 0·1 M-tris, pH 7·0, if RNA is to be prepared from the virus, *vide infra*). After dialysis about 3·5 ml. of phage suspension is recovered. The total concentration of virus at this stage is about 80 mg/ml. of which 15 to 20% are active plaque formers.

(d) *Preparation of MS2 RNA*

All operations are carried out at 4°C.

To a cold suspension of purified MS2 at about 25 mg/ml. in 0·1 M-tris, pH 7·0, is added an equal vol. of cold, redistilled phenol (Gierer & Schramm, 1956) saturated with the same buffer. The mixture is shaken for 1 min on a Vortex mixer, and the emulsion then broken by centrifugation at 6000 *g* for 10 min. The aqueous layer is removed and added to an equal vol. of phenol and the extraction process repeated.

To improve the yield of MS2 RNA, a volume of tris equal to the original volume of phage suspension is added to the phenol layer from the first extraction, shaken and spun as before. The aqueous layer is then used to extract the phenol layer from the second extraction. This aqueous layer is then combined with the initial aqueous layer, and the RNA precipitated by the addition of 2 vol. cold ethanol. After several hours the precipitate is spun out at 6000 *g* for 15 min. The supernatant solution is discarded and the precipitate taken up in 0·1 M-tris, pH 7·0. The RNA is precipitated twice more in the same way. The final RNA precipitate is taken up in one-half to one-third the initial vol. of phage suspension and spun at 27,000 *g* for 30 min to remove insoluble material. The supernatant solution is then dialysed for short times against several large volumes of tris to remove residual phenol. The final RNA preparation, containing about 10 mg/ml., is divided into small portions and stored at −70°C. The final preparation accounts for 80 to 90% of the RNA of the initial virus suspension.

(e) *Methods of purine and pyrimidine analysis*

Degradation of the RNA was accomplished either with 1 N-HCl at 100°C for 1 hr (Smith & Markham, 1950), or with 0·3 N-KOH at 37°C for 18 hr (Marrian, Spicer, Balis & Brown, 1951). The mixture of free purines and pyrimidine nucleotides resulting from the

† In recent preparations the buffer has also contained 0·01 M-EDTA. The presence of EDTA during the ammonium sulfate precipitation and subsequent steps increases the stability of the virus.

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acid hydrolysis was separated by descending chromatography in the methanol-ethanol-HCl-water solvent of Kirby (1955), using Whatman no. 1 filter paper. The mixture of nucleotides resulting from alkaline hydrolysis was first passed through a Dowex 50W-X1 column in the ammonium form to remove potassium, and the effluent lyophilized. The nucleotides were taken up in 0·04 M-citrate, pH 3·5, and separated by paper electrophoresis in this buffer at 40 v/cm (Davidson & Smellie, 1952).

In all cases only four ultraviolet-absorbing spots resulted. These spots were eluted with 0·01 N-HCl, and the amount of material determined from the ultraviolet absorption in this solvent, after correction for the appropriate blanks. Spectra were taken on the Beckman DK2 recording spectrophotometer. The extinction coefficients used were those given at 260 mμ by Beaven, Holiday & Johnson (1955).

In one instance in which recovery was determined, 96% of the ultraviolet absorbancy of the alkaline digest was recovered as nucleotides after electrophoresis.

(f) *Light scattering*

All light scattering measurements were made on a Brice-Phoenix light scattering photometer, model 1000 D, made by Phoenix Precision Instruments, Inc., using the 4358 Å Hg line. Solutions were first cleaned of dust by filtration through type HA Millipore filters (Millipore Filter Co., Bedford, Mass.).

3. Properties of MS2

The sedimentation coefficient of MS2, $S_{20,w}$, is 81 s in 0·1 M-NaCl plus 0·01 M-tris, pH 7·6, at 120 μg/ml.

In the electron microscope MS2 appears to be a polyhedral object about 26 mμ in diameter (A. Hodge, in preparation).

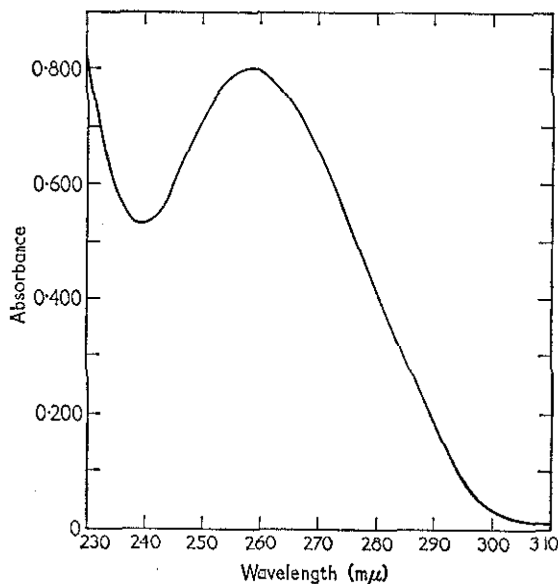

Fig. 1. Ultraviolet absorption spectrum of purified MS2 at a concentration of 99 μg/ml.

The dry weight of the virus was obtained from samples desiccated at room temperature and then dried at 105°C *in vacuo* over P_2O_5. From this measurement, the specific absorption of the virus in 0·1 M-NaCl plus 0·01 M-tris, pH 7·6, was found to be 8·03/mg/ml. at 260 mμ on the Beckman DK2. An absorption spectrum of the purified virus taken on the DK2 is shown in Fig. 1.

A light scattering envelope of the virus is shown in Fig. 2. The refractive increment of the virus was measured to be 0·191 ml./g at 436 mμ on a Brice-Phoenix differential refractometer. Concentrations were determined spectrophotometrically. The light scattering was performed at several concentrations; within experimental error the intercept was independent of concentration. From the intercept, the particle weight is $3{\cdot}6 \times 10^6$.

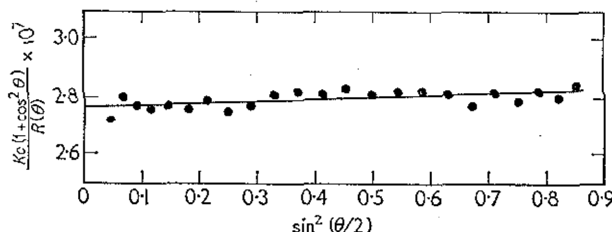

Fig. 2. Light scattering by MS2 at a concentration of 82 μg/ml.

The virus bands at a density of 1·46 in dilute suspensions in a CsCl density gradient. In the more concentrated suspensions involved in the preparation of large quantities of MS2, the virus bands at a density of about 1·38.

The virus was found to contain 28·5 mg phosphorus per gram. Assuming that phosphorus is found only in the nucleic acid, MS2 is 31·5% RNA (calculated as the hydrogen form of the RNA).

4. Properties of MS2 RNA

(a) *Composition*

The averages of several analyses of the purine and pyrimidine composition of MS2 RNA are shown in Table 1 for the two methods used. The base composition is seen to be approximately equimolar with slightly smaller amounts of adenine and slightly greater amounts of guanine.

TABLE 1

Purine and pyrimidine composition of MS2 RNA

Mode of analysis	Mole fractions			
	Adenine	Uracil	Cytosine	Guanine
Acid hydrolysis, chromatographic separation	0·232	0·248	0·253	0·267
Alkaline hydrolysis, electrophoretic separation	0·224	0·256	0·245	0·275

(b) *Hydrogen bonding*

MS2 RNA reacts slowly with 1·8% formaldehyde at 37°C (Fig. 3). The reaction requires 12 to 14 hours to come to completion (as judged spectrophotometrically), during which time the absorbancy at the maximum increases 25%. This slow rate of reaction would seem to indicate a strong hydrogen bonding in the RNA (Hall & Doty, 1959). The reaction of formaldehyde with single-stranded ϕX DNA under the same conditions is complete in 7 hours (Sinsheimer, 1959*b*).

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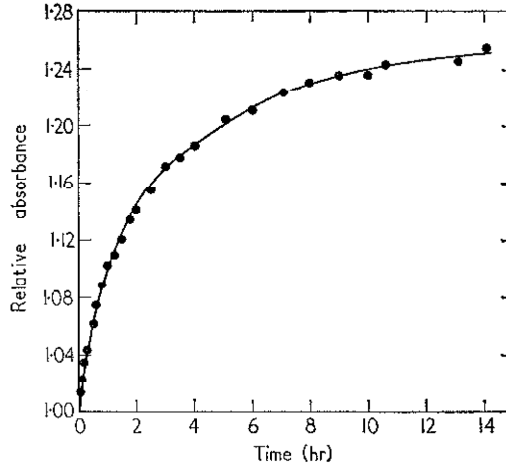

FIG. 3. Reaction of MS2 RNA in 0·1 M-Na phosphate, pH 7·0, with 1·8% formaldehyde at 37°C. Concentration of RNA = 33 μg/ml. The relative absorbance is that at 258 mμ, relative to that before addition of formaldehyde.

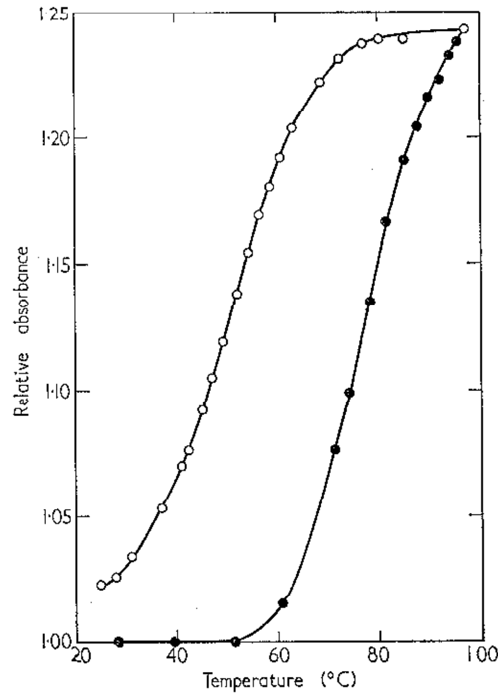

FIG. 4. Variation of the ultraviolet absorption of MS2 RNA at 257 mμ with temperature. The relative absorbance is the absorbance at the indicated temperature, relative to the absorbance at 5°C.
●—● RNA in 0·14 M-NaCl+0·01 M-tris, pH 7·0; ○—○ RNA in 0·02 M-NaCl+0·001 M-tris, pH 7·0.

The loss of ordered structure in MS2 RNA upon heating was followed by measurements of absorbancy, with the Beckman DK2, of RNA samples in jacketed cells at various temperatures. The transition occurs only at relatively elevated temperatures at moderate salt concentrations. In 0·14 M-NaCl plus 0·01 M-tris, pH 7, the midpoint of the transition, T_m, is 76°C (Fig. 4). This high T_m is presumably not a consequence of the presence of divalent cations as impurities, because addition of 3×10^{-4} M-EDTA does not alter the T_m. In addition the T_m is not altered by the addition of 0·01 M-Mg^{2+}.

The transition is reversible as judged by the T_m. Upon heating a sample of RNA to 95°C for 10 minutes, cooling and remelting, the same T_m is observed.†

In 0·02 M-NaCl the T_m is reduced to 49°C (Fig. 4).

(c) *Light scattering observations*

The particle weight of MS2 RNA has been measured by light scattering. The refractive increment was taken as 0·194 ml./g as measured by Northrop & Sinsheimer (1954) for TMV RNA.

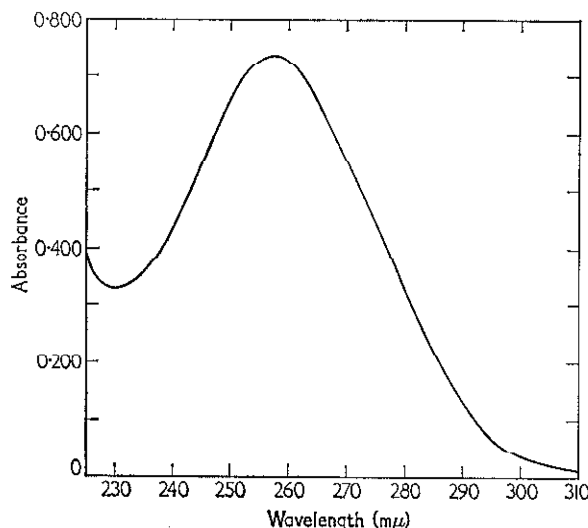

FIG. 5. Ultraviolet absorption spectrum of MS2 RNA in 0·05 M-tris, pH 7·0, at 25°C. Concentration of RNA = 29 μg/ml.

The extinction coefficient of the RNA at 260 mμ was found to be 8600 per mole of phosphorus in 0·05 M-tris, pH 7·0, at 25°C in the Beckman DU spectrophotometer. From the base composition of the RNA reported above the specific absorption of the RNA under these conditions is thus 25·1/mg/ml. for the hydrogen form. An absorption spectrum of the RNA taken in the DK2 is shown in Fig. 5.

Zimm plots for the RNA in 0·05 M-NaCl and in 0·003 M-tris are shown in Fig. 6 and Fig. 7, and light scattering envelopes (extrapolated to zero concentration) for the RNA at four different salt concentrations are given in Fig. 8. All light scattering measurements were performed at 6°C at pH 7·0.

† Variable amounts of residual hyperchromicity have been observed after heating and cooling, the minimum amount being about 1%. It is not known whether this is due to hydrolysis occurring at the high temperatures, or whether some of the effect is due to irreversibility in the transition.

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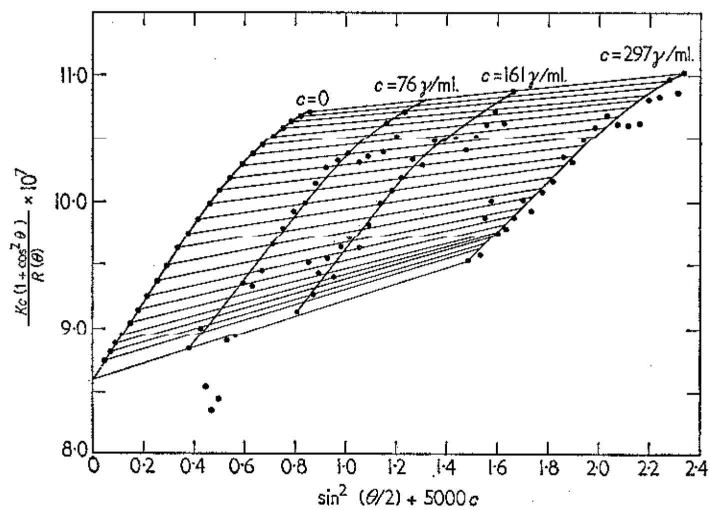

FIG. 6. Light scattering of MS2 RNA in 0·05 M-NaCl + 0·003 M-tris, pH 7·0, at 6°C.

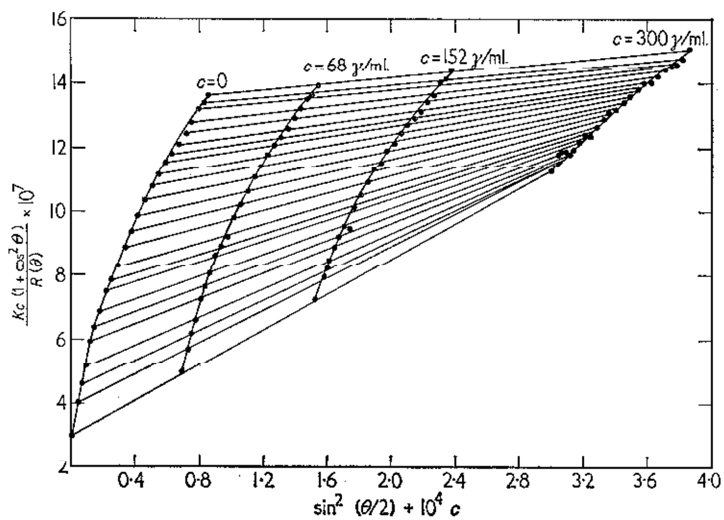

FIG. 7. Light scattering of MS2 RNA in 0·003 M-tris, pH 7·0, at 6°C.

In 0·2 M-NaCl† the observed particle weight of the RNA is $1{\cdot}05 \times 10^6$. This molecular weight accounts for 93% of the phosphorus content of the whole virus. MS2 thus contains probably only one molecule of RNA per phage particle.

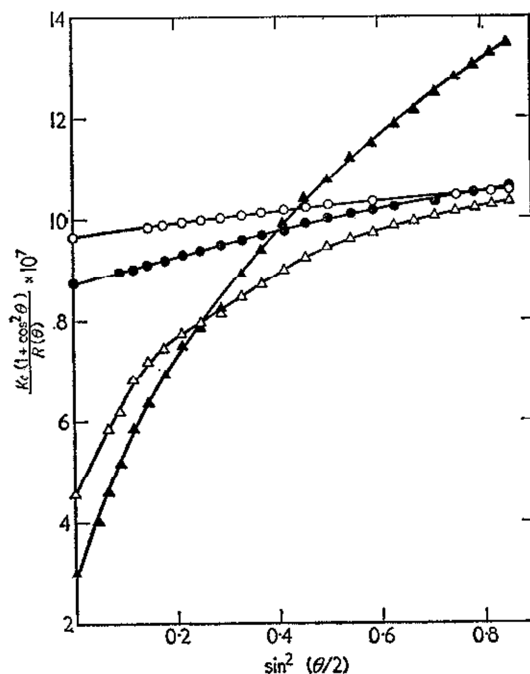

FIG. 8. Light scattering envelopes (extrapolated to zero concentration) of MS2 RNA at different salt concentrations at 6°C.
○——○, in 0·2 M-NaCl+0·01 M-tris, pH 7·0; ●——●, in 0·05 M-NaCl+0·003 M-tris, pH 7·0; △——△, in 0·02 M-NaCl+0·003 M-tris, pH 7·0; ▲——▲, in 0·003 M tris, pH 7·0.

The radius of gyration in 0·2 M-NaCl is 160 Å. It is evident from this that the molecule is very tightly coiled. For a random coil the radius of gyration should vary as $M^{0{\cdot}5}$ (Tompa, 1956). For MS2 RNA $(\sqrt{M})/R_G$ (M is the molecular weight in molecular weight units, R_G the radius of gyration in Å) is 6·3 under these conditions. This may be compared with a value of 3·8 for TMV‡ RNA in 0·1 M-Na phosphate at 6°C (Boedtker, 1960), and with a value of 3·0 for single-stranded ϕX DNA in 0·2 M-NaCl at 37°C (Sinsheimer, 1959*b*).

In 0·05 M-NaCl the observed molecular weight is $1{\cdot}15 \times 10^6$, and the radius of gyration is 250 Å ($(\sqrt{M})/R_G = 4{\cdot}3$). Furthermore, exposure of the RNA to 0·05 M-NaCl produces an expansion of the molecule which is largely irreversible under these conditions, since the radius of gyration remains almost unchanged upon bringing the NaCl concentration to 0·2 M. Under these conditions $(\sqrt{M})/R_G$ is close to that found for TMV RNA.

† A Zimm plot in 0·2 M-NaCl cannot be conveniently given because of the small radius of gyration of the particle. The total change in the amount of scattering between 0° and 135° at any concentration is only about 10% of the total amount of scattering. A light scattering envelope is given in Fig. 8.

‡ Abbreviation used: TMV=tobacco mosaic virus.

Upon lowering the salt concentration to 0·02 M-NaCl, however, the molecular weight and radius of gyration change considerably (Fig. 8). From the limiting behavior of the light scattering envelope the molecular weight is found to be $2{\cdot}2 \times 10^6$ and the radius of gyration 900 Å. Further lowering of the salt concentration to 0·003 M-tris increases the observed molecular weight to $3{\cdot}3 \times 10^6$ and the radius of gyration to 1250 Å (Fig. 7). Under these conditions the RNA thus exists in an aggregated state. The molecular weight of the RNA has also been measured to be 2×10^6 in 0·02 M-NaCl at 20°C, and in 0·02 M-NaCl at 6°C at pH 8·2 (3×10^{-3} M-tris buffer).

The aggregation of the RNA is largely reversible. Upon bringing the salt concentration from 0·003 M-tris to 0·05 M or 0·2 M-NaCl, the molecular weight and radius of gyration return approximately to the values measured in 0·05 M-NaCl without prior exposure to low ionic strength.

(d) *Sedimentation velocity*

The sedimentation properties of MS2 RNA were studied in the model E ultracentrifuge using the ultraviolet optics.

A densitometer tracing from a sedimentation in 0·1 M-tris, pH 7, at 5°C is shown in Fig. 9. Approximately 70% of the material sediments in a fairly sharp boundary while

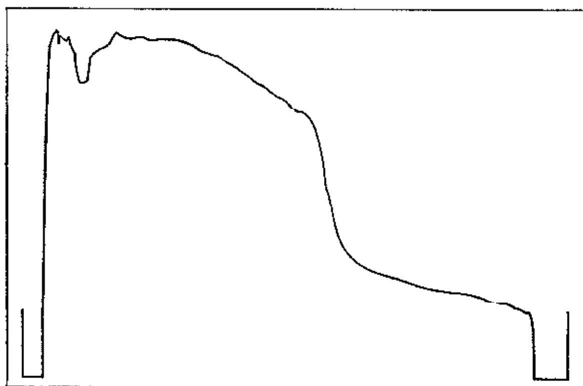

FIG. 9. Sedimentation of MS2 RNA at 36 μg/ml. in 0·1 M-tris, pH 7·0, at 5°C. Densitometer tracing of a picture taken after 28 min of centrifugation at 56,100 rev./min. Sedimentation is from left to right.

the rest of the material trails. More recent preparations have sedimented with up to 90% of the material in the leading boundary. Sedimentation coefficients below refer to this leading boundary; the average coefficient of the trailing material is about 70% that of the leading material.

In 0·1 M-tris, pH 7·0, the sedimentation coefficient of the RNA, $S_{20,\mathrm{w}}$, is 27 s.

After reaction with formaldehyde with disruption of intramolecular hydrogen bonds, the sedimentation coefficient is reduced to 14 s (in 0·1 M-Na phosphate, pH 7, plus 1·8% formaldehyde).

In 0·2 M-NaCl the $S_{20,\mathrm{w}}$ is 31, while in 0·02 M-NaCl it is 27 s. Both of these coefficients are calculated from measurements at 5°C. It will be recalled that in the latter case the light scattering studies have shown the RNA to have twice the molecular weight and a much larger radius of gyration than in the former case. It might be noted that the sedimentation boundary obtained in 0·02 M-NaCl is fairly sharp (Fig. 10).

Upon increasing the salt concentration of an RNA sample from 0·02 M to 0·2 M-NaCl the $S_{20,w}$ becomes 28·5 s (again calculated from measurement at 5°C), in agreement with the light scattering observation that the monomer undergoes an irreversible expansion upon exposure to low salt concentration.

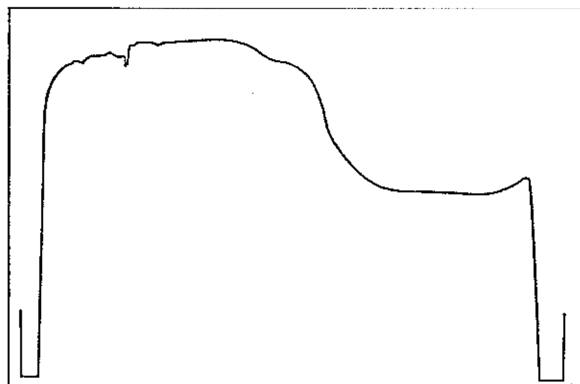

FIG. 10. Sedimentation of MS2 RNA at 36 μg/ml. in 0·02 M-NaCl + 0·002 M-tris, pH 7·0, at 5°C. Densitometer tracing of a picture taken after 24 min of centrifugation at 56,100 rev./min. Sedimentation is from left to right.

5. Discussion

The high T_m, indicating a strong hydrogen bonding, the small radius of gyration and high sedimentation coefficient, indicating a compact structure, and the ability to aggregate in low salt suggest that MS2 RNA has an unusual structure.

The sedimentation coefficient, $S_{20,w}$, of TMV RNA of molecular weight $2 \cdot 0 \times 10^6$ (Boedtker, 1960) is 34 s in 0·2 M-NaCl under the same conditions used for MS2 RNA, while in 0·02 M-NaCl it is 27 s (unpublished observations). Thus MS2 RNA sediments more rapidly at the higher salt concentration than expected on the basis of the molecular weight difference at this salt concentration, even after exposure of MS2 RNA to 0·02 M and return to 0·2 M-NaCl, with concurrent expansion of the molecule. In 0·02 M-NaCl the two species have the same molecular weight by light scattering and the same $S_{20,w}$.

The T_m for TMV RNA in 0·15 M-NaCl is 49°C (Boedtker, 1960); however, this RNA has a lower GC content (Knight, 1952). The higher T_m of MS2 RNA may be due to both GC content and the approximately equimolar base composition, which may allow a greater number of hydrogen bonds to be formed. Extensive intra-strand hydrogen bonding could explain the compact structure of the RNA.

The aggregation of the RNA in low salt is surprising. The preference of the RNA to form intermolecular bonds exists only at low ionic strength, since aggregation is reversible upon return to high ionic strength. Conceivably at low ionic strength the pKs of some groups are raised sufficiently to acquire a positive charge at neutral pH and permit formation of an aggregate through electrostatic interactions; however, the aggregation in 0·02 M-NaCl also occurs at pH 8·2. In this connection it has yet to be demonstrated that the RNA is homogeneous; heterogeneity might favor an intermolecular aggregation.

It might be noted that no change in light scattering molecular weight is observed upon going from 0·2 M to 0·02 M-NaCl in the case of ϕX174 DNA (Sinsheimer, 1959*b*) or with TMV RNA (Denhardt & Yarus, unpublished observations).

MS2 RNA, unlike TMV RNA, does not precipitate in 1 M-NaCl at high RNA concentrations.

There is a discrepancy between the apparent size of the MS2 particle in the electron microscope, the buoyant density in CsCl and the molecular weight of the particle. A particle 26 mμ in diameter with a uniform density of 1·46 would have a molecular weight of $8 \cdot 0 \times 10^6$. However, the $S_{20,w}$ of the particle is correspondingly low; from the sedimentation equation, $S = M(1-\bar{v}\rho)/Nf$ (assuming the frictional coefficient f is given by $6\pi\eta R$, where R is the radius of the particle measured in the electron microscope, and that the partial specific volume $\bar{v}$ is the reciprocal of the density in CsCl), a molecular weight of $3 \cdot 8 \times 10^6$ is calculated. A likely explanation of this phenomenon is that portions of the virus may be hollow and permeable to CsCl.

It is of interest to compare the properties of MS2 with other small RNA viruses. Physical properties of the small plant virus bromegrass mosaic virus have been reported by Kaesberg (1959) and by Bockstahler & Kaesberg (1962), and those of broad bean mottle virus have been reported by Yamazaki, Bancroft & Kaesberg (1961). These viruses contain RNA of molecular weight close to one million. The former has an $S_{20,w}$ of 86 and a molecular weight of $4 \cdot 6 \times 10^6$, the latter an $S_{20,w}$ of 85 and a molecular weight of $5 \cdot 2 \times 10^6$; both viruses contain 22% RNA. MS2 thus has a lower molecular weight and higher percentage of RNA than these viruses, while containing approximately the same absolute amount of RNA per particle. In addition bromegrass mosaic virus has a diameter of 28 mμ by X-ray scattering and a partial specific volume of 0·708 ml./g. Thus the same type of discrepancy between size, molecular weight and density exists with this virus as with MS2; a particle with a density of 1·41 (the reciprocal of $\bar{v}$) and a diameter of 28 mμ would have a molecular weight of $9 \cdot 8 \times 10^6$. It is interesting in this regard that bromegrass mosaic virus has a central cavity of more than 10 mμ in diameter (cited in Bockstahler & Kaesberg, 1962), which could explain the discrepancy in this case.

The determination of the base composition and of the extinction coefficient of MS2 RNA were performed by Mr. John Sedat. This research was supported in part by research grant RG6965 and training grant 2G-86 from the U.S. Public Health Service.

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DISCUSSION

Since the isolation of an RNA-containing bacteriophage was first reported by Loeb and Zinder (1), the isolation of several similar RNA phages has been reported (2-6). In addition to the present studies on MS2, however, only the physical properties of RNA phages R17 (7-9) and fr (10) have been investigated in any detail. R17 and MS2 appear to be very similar but not identical (7-9, 11-14). The agreement among the various groups concerning the physical properties of R17 and MS2 and of their RNA's has been discussed by Gesteland and Boedtker (9); accordingly, this discussion will be limited to avoid redundancy. The physical properties of fr are similar to those of MS2 and R17.

The T_m of 76° in 0.15 M NaCl found for MS2-RNA is evidently an artefact caused by divalent cation contamination. RNA isolated from virus prepared in the presence of 0.01 M EDTA has a T_m of 63° in this solvent or in 0.15 M NaCl-0.015 M Na citrate. This value still appears significantly higher than the T_m of 58° found for R17-RNA (8,9). The melting properties of MS2-RNA will be further discussed in Part IV.

The preparations of RNA showing this lowered T_m still show the aggregation phenomenon, however, both at 6° and at 20°. Gesteland and Boedtker (9) have found that

complete removal of divalent cations with EDTA from their R17-RNA preparations removes any tendency of their preparations to aggregate. Since our RNA preparations do appear to contain residual divalent cation contaminants (see Part V), these may play a role in this phenomenon. Whether the tendency to aggregate has any significance is unknown.

Due to systematic errors in the calculations, all physical properties quoted in this publication refer to the sodium salt of the RNA rather than to the hydrogen form as stated; these include percentage RNA of the virus, and extinction coefficient and molecular weight of the RNA.

The specific absorption at 260 mμ of 25.1/mg/ml. found for MS2-RNA appears to be too large. Mitra et al. (8) found a value of 23.3/mg/ml. for R17-RNA; since this value evidently refers to the hydrogen form of the RNA, it is 13% lower than our value for MS2-RNA. The specific absorption at 258 mμ of 22.5/mg/ml. for TMV-RNA (quoted in ref. 9) is 10% lower than our value for MS2-RNA, and in view of the respective wavelengths and base compositions we would expect the specific absorption of MS2-RNA to be lower. Furthermore, from the extinction coefficients at 260 mμ of the nucleotides (15) and the hyperchromicity shown by the RNA at this wavelength at 100°C, we again suspect the extinction coefficient to be high by 10-12% (16, 17).

Since the extinction coefficient enters directly into the calculation of molecular weight from the light scattering data, if the extinction coefficient is 12% high, the estimate of the molecular weight will be too large by the same amount.

The molecular weight of the intact MS2-RNA molecule will be larger than the weight average molecular weight measured for our heterogeneous RNA preparations. A reasonable estimate of the decrease in weight average molecular weight due to random degradation in our preparations is 10-12%.

Since these two effects act in opposite directions and are of the same magnitude, the best estimate from light scattering for the molecular weight of the sodium salt of the undegraded MS2-RNA molecule remains 1.05×10^6. Three other estimates of this value are available of varying reliability, but in general agreement with the light scattering results. We have measured the intrinsic viscosity of our MS2-RNA preparations to be 0.30 dl./gm in 0.2 M NaCl at 5°C. Taken together with the sedimentation coefficient under these conditions, the Scheraga-Mandelkern equation gives a molecular weight of 1.1×10^6 (assuming $\beta = 2.25 \times 10^6$). Among other factors, this estimate is also subject to the same considerations of errors in extinction coefficient and heterogeneity. In

dimethylsulfoxide MS2-RNA sediments as does an RNA of molecular weight 1.1×10^6 (Part V). Finally, the RNA molecular weight calculated from the phosphorus content and light scattering particle weight of the entire virus particle is 1.15×10^6.

MS2-RNA is stable to heating and denaturing conditions (Part V), reinforcing the conclusion reached in the publication that all of the RNA of the virus is found in a single covalently-bound polynucleotide chain, and in agreement with the conclusions of Gesteland and Boedtker (9) for R17-RNA.

The sedimentation coefficient found for R17-RNA (8,9) in 0.1 M salt is similar to our value for MS2-RNA. A discrepancy exists in the sedimentation coefficients in 0.01 or 0.02 M salt, however, where our values for MS2-RNA (measured at $5^{o}C$) are higher than those found for R17-RNA (measured at 5° or at $20^{o}C$). This discrepancy is partially resolved in that our sedimentation coefficients at this salt concentration are similar to those found for R17-RNA when the measurements are made at 20 or $25^{o}C$. These results will be discussed in Part V.

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PART II

STUDIES ON AN INFECTIVITY ASSAY FOR MS2-RNA

INTRODUCTION

Infectivity assays have been described for the free nucleic acids of many bacterial, plant, and animal viruses, both DNA- and RNA- containing. The availability of a good infectivity assay can be very useful in the study of viral nucleic acids, for as most often used it is a test for the complete genome of the virus.

To assay MS2-RNA we originally used the infectivity assay developed by Guthrie and Sinsheimer (1,2) for the single-stranded DNA of phage ØX174, with the sole modification that the RNA was in 0.05 M tris pH 7 instead of pH 8.1 (3,4). The efficiency of this assay varies from about 2×10^{-8} to 5×10^{-7} infections per RNA molecule and is difficult to reproduce. Since our viral stocks usually contain about 20% active plaque formers, this efficiency must be multiplied by five to express it as RNA infections per phage plaque former as is done by several authors. This result is of the same magnitude or somewhat better than the results first reported by other investigators working with phage RNA (5-8).

The report of Ginoza and Vessey (9) led us to re-examine the MS2-RNA infectivity assay in order to improve it.

MATERIALS AND METHODS

Materials

PA and PAM media are as in Guthrie and Sinsheimer (2) except that these media contain 1 g glucose/l. 3XD is as in Fraser and Jerrel (10). Techniques involved in plating of phage are as in Part I except that bacterial strain A19 was often used for seed bacteria.

Bacterial strains A19 (which lacks RNase I) and Q13 (which lacks both RNase I and polynucleotide phosphorylase) (11) were obtained from Dr. R. F. Gesteland.

EDTA used for conversion of the cells to protoplasts is 4% Na_2EDTA in water. Crystalline lysozyme from Sigma is dissolved in 0.25 M tris, pH 8.1, at 2 mg/ml.; such a solution should not be used for longer than two weeks. BSA is a 30% sterile solution containing 0.1% NaN_3 as a preservative obtained from Armour Pharmaceutical Company, Kankakee, Ill.

Tris-Cl buffer, pH 7, is made by neutralization of a 1 M solution of Sigma Trizma Base with concentrated HCl to pH 7, at which time the temperature of the solution is 35-37°C.

Infectivity Assay

The infectivity assay is a modification of that of Guthrie and Sinsheimer (2). Conversion, infection, and incubation periods are defined below. Cells and

protoplast stocks are counted with a Petroff-Hausser counter.

Cells to be converted to protoplasts are grown to 3-5 x 10^8/ml. in 3XD and collected by centrifugation at room temperature. Conversion to protoplasts is performed at room temperature. For each 20 ml. of original cell suspension are added in order: 0.35 ml. of 1.5 M sucrose, 0.17 ml. of 30% BSA, 0.02 ml. of lysozyme solution, 0.04 ml. of 4% EDTA. For conversion times on one min. or longer 9.6 ml. of PA medium is added immediately, followed by 0.2 ml. of 10% $MgSO_4$ after the desired conversion time. For conversion times of one min. or less 9.6 ml. of PAM medium is added after the desired conversion time. The resultant protoplast stock is routinely chilled before use.

The bacterial strain routinely used for the preparation of protoplast stocks has been _E_. _coli_ K12, W6. These cells and the protoplasts made from them are resistant to the mature MS2 virus, thus eliminating re-infection during the assay. Some experiments have been conducted with the _E_. _coli_ strains C3000, A19, and Q13, all of which are susceptible to the mature virus; protoplast stocks made from them are also susceptible to the virus. In this case, then, either infective centers must be plated or, if progeny phage are assayed, the time of incubation must be carefully controlled. In the latter

case the progeny phage titer is not strictly proportional to the concentration of the input RNA, although the non-linearity of the assay is not extreme.

Infectivity assays are normally performed by adding 0.4 ml. of the protoplast stock to 0.4 ml. of the RNA suspension in the desired buffer (usually 0.05 M tris, pH 7). After the selected infection interval (optimally about 30 sec.) this mixture is diluted with 3.2 ml. of PAM medium. Following incubation, the infected protoplasts are lysed by one of three methods: freeze-thawing once, addition of a few drops of $CHCl_3$ followed by vigorous mixing, or dilution into distilled water for 10 min. All three methods give comparable results. The released phage are then titered.

If, instead, it is desirable to assay for infected protoplasts, the best results are obtained by following the above procedure, then plating by the method of Guthrie and Sinsheimer (2) after a 10-15 min. incubation (intra-cellular phage do not appear until 25-30 min. after infection).

We have routinely used B-D Cornwall Continuous Pipetting Outfits (Becton, Dickinson, and Co., Rutherford, N. J.) for dispensing both protoplast stock and PAM medium when large numbers of samples are involved. For the modified assay where the time interval between these two

operations is only 30 sec., use of these outfits by two persons working in series greatly facilitates the operation and leads to better reproducibility.

The results are often expressed as output phage/RNA molecule or as infected protoplasts/RNA molecule. When progeny phage are assayed the latter figure is arrived at from the burst size measured in single burst experiments.

RNA Preparations

MS2-RNA is prepared as in Part I. The concentration of RNA is expressed in molecules/ml., found from the optical density of the preparation and the physical data in Part I: 2.3×10^{13} RNA molecules/absorbancy unit at 260 mμ.

TMV-RNA and *E. coli* ribosomal RNA are prepared as in Part V.

Single Burst Experiments

Single burst experiments are performed by diluting the RNA to a low concentration and placing 0.4 ml. aliquots into the desired number of tubes. Each tube is assayed in the regular fashion. After incubation and lysis the tubes are assayed for phage; the RNA concentration is adjusted so that phage are present in about 10% of the tubes.

Abbreviations Used

BSA = bovine serum albumin

TMV = tobacco mosaic virus

PFU = plaque forming unit

Definitions

For ease of presentation, we will define the following three terms:

Conversion Period: the time interval in which bacterial cells are converted to protoplasts. This step is considered to begin with the addition of EDTA to the cells and to end with the addition of Mg^{++}.

Infection Period: the time interval in which infection of the protoplasts by the RNA takes place. It begins with the addition of the protoplast stock to the RNA suspension, and is considered to end with the dilution of this mixture with PAM medium. Similarly, infection mixture and infection tube refer to this mixture of RNA and protoplast stock (0.8 ml. volume).

Incubation Period: the time interval in which phage production occurs in the infected protoplasts. It is considered to begin with the dilution of the infection mixture with PAM medium and to end with the chilling and/or lysis of the mixture. Incubation tube refers to this mixture (4.0 ml. volume).

RESULTS

Preparation of Protoplast Stocks

We have consistently observed that short times of conversion of cells to protoplasts (of the order of 30 seconds) give better stocks, and that the omission of BSA from the protoplast stocks decreases the infectivity of RNA preparations by roughly an order of magnitude. The data in Table II-1 illustrate these observations.

Protoplast stocks prepared with a short conversion time contain less than 50% completely rounded forms when examined microscopically, but 90% or more of the cells can be seen to have been affected by the lysozyme treatment as evidenced by partial rounding. These stocks are stable for at least 24 hours in the cold.

When the infection period is shorter than in Table II-1 (see below) the time of conversion is still optimal at about 30 seconds. In this case the BSA effect is more pronounced, however.

Attempts to prepare protoplast stocks using a 30% solution of BSA made with Armour or Sigma Fraction V powder have not been reproducible. Usually such stocks are much less efficient than those prepared from the same cells using the 30% solution obtained from Armour.

When the cell concentration of W6 is allowed to go

Table II - 1

Effect of Conversion Time and BSA on Protoplasts Stocks

Experiment	Time of Conversion (min.)	Phage/RNA x 10^4	
		+BSA	-BSA
1	1	1.7	0.28
	3	1.6	0.24
	6	1.4	0.27
	10	0.6	0.17
	20	0.6	0.15
2	0.5	1.4	0.16
	1	0.6	0.11

The RNA solutions at 10^{10}/ml. in 0.05 M tris pH 7 were mixed with an equal volume of the protoplast stocks at 35°, and after 10 min. the infection mixture was diluted five-fold with PAM medium. The samples were then incubated for 90 min. at 35°. The concentration of the protoplast stock was 6×10^8 in experiment 1 and 7×10^8 in experiment 2.

beyond 5×10^8/ml. the resulting protoplast stocks are definitely inferior. Unless the cell concentration is followed carefully, more consistent results are obtained by removing the cells at $3\text{-}4 \times 10^8$/ml.

The Infection and Incubation Periods

The length of the infection period is critical for optimum results. The optimum has varied in time as well as sharpness for different protoplast stocks; the results of varying the length of the infection period with several stocks is shown in Figure II-1. As can be seen, this parameter must be closely controlled for reproducible results. The sharpness of the optimum appears to be correlated to some extent with the concentration of the protoplast stock: less concentrated stocks give a broader plateau. Figure II-1 shows this effect. The decline in progeny phage titer as the infection period is lengthened is almost certainly due to a loss of infected protoplasts. Single burst experiments show no significant difference in burst size between infection times of 30 seconds and 10 minutes.

The optimum for the ratio of the volume of RNA solution to the volume of protoplast stock mixed with it to initiate infection is fairly broad around equal volumes of the two. Maximum phage production requires dilution of the infection mixture at least five-fold with PAM medium;

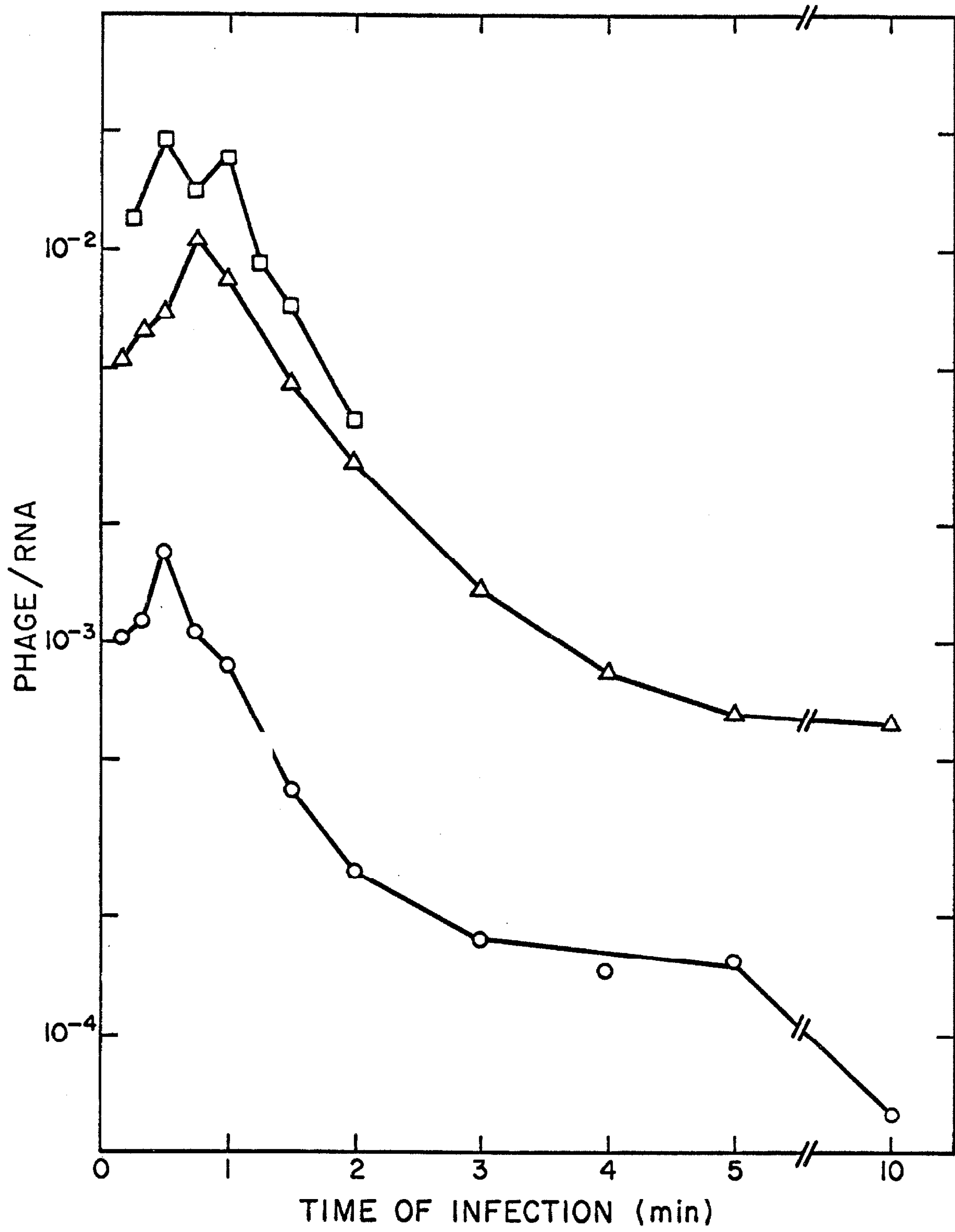

Fig. II - 1

Figure II-1 Effect of the Length of the Infection Period on Phage Titer

MS2-RNA at 10^9/ml. (experiments 1 and 2) or 10^{10}/ml. (experiment 3) in 0.05 M tris was mixed with an equal volume of protoplast stock. At the times indicated in the figure the infection mixture was diluted five-fold with PAM medium. Infection and incubation were at 37°C. All protoplast stocks were prepared with a conversion time of 30 seconds.

O-O Experiment 1, concentration of protoplast stock = 1.2×10^9/ml. Incubation for 90 min.

Δ-Δ Experiment 2, protoplast stock prepared from W6 grown to 4.3×10^8/ml. Incubation for 90 min.

□-□ Experiment 3, concentration of protoplast stock = 5.4×10^8/ml. Incubation for 3 hours.

further dilution is without effect.

The optimum temperature for incubation is 36 to 38°C. If the infection is performed at 30°C (followed by incubation at 37°C), the optimum time of infection is longer than when the infection is at 37°C (as in Figure II-1). If the time of infection is optimal for infection at either 30°C or 37°C, and incubation is at 37°C, the phage titer produced is independent of the temperature during the infection process. Thus, the optimum in the incubation temperature is probably due to differences in the burst size.

Maximum phage production requires 2.5 to 3 hours of incubation at 37°C. The phage titer is then stable for at least another two hours (Figure II-2).

Tris and phosphate buffers of varying ionic strength as the RNA solvent have been investigated to some extent. Buffer concentrations above 0.05 M are sharply inhibitory to the assay. Results using buffer concentrations from 0.001 M to 0.05 M have not been completely satisfactory, but we usually observe an optimum at 0.005 to 0.01 M buffer with little difference between phosphate and tris buffers. The results of two experiments showing this effect are given in Table II-2, and are included primarily to illustrate the magnitude of the differences involved.

Three pH's have been tested for the RNA solvent. No

Table II - 2

Influence of the Buffer Concentration of the RNA Solvent on Phage Titer

Experiment	Buffer Concentration (M)	Phage/RNA x 10^3	
		Tris	Phosphate
1	0.01	4.7	4.7
	0.05	2.6	1.2
	0.1	0.7	0.1
	0.2	---	10^{-6}
2	0.001	6.2	6.2
	0.005	9.2	8.5
	0.01	10.4	6.2
	0.05	7.0	2.4

MS2-RNA at 10^{10}/ml. in the indicated buffer at pH 7.0 was mixed with an equal volume of the protoplast stock. After 30 seconds the infection mixture was diluted five-fold with PAM medium and incubated at 37°C for 3 hours.

difference was found between 0.05 M tris at pH 7.0 or pH 8.1. 0.05 M Na acetate at pH 5.0 was an order of magnitude less efficient than 0.05 M tris at pH 7.0.

The presence of up to 10% of the organic solvents formamide, dimethylformamide, or dimethylsulfoxide in the RNA solvent has little effect on the assay (not more than 30% inhibition).

A Modified Infectivity Assay for MS2-RNA

The following publication has been included as a brief summary of the modified assay for MS2-RNA evolved from these studies. This article is reproduced from the Journal of Molecular Biology (1964), vol. 10, p. 422, by permission of Academic Press Inc. (London) Ltd.

A Modified Method of MS2-RNA Assay

JAMES H. STRAUSS, JR.

A modification of the previously described assay method (Davis, Pfeifer & Sinsheimer, 1964) partly inspired by the suggestions of Ginoza & Vessey (1964, Abst FC5, *Eighth Annual Meeting of the American Biophysical Society*), provides a marked increase in the efficiency of RNA infection.

A bacterial protoplast stock of *Escherichia coli* K12 (strain W6) is prepared as described by Guthrie & Sinsheimer (1963), except that PAM medium† is added 30 seconds after addition of the EDTA to the resuspended cells. This stock, which contains about 10^9 protoplasts/ml., may be chilled and used for at least 24 hours without loss of efficiency.

For assay, 0·4 ml. of protoplast stock (at room temperature) is added to an equal volume of an RNA solution, in a 37°C water bath, in 0·05 M-tris buffer, pH 7·0 (measured at 37°C). Thirty seconds later, 3·2 ml. of PAM medium is added. After 2·5 hours incubation at 37°C, the protoplasts are disrupted by addition of a few drops of chloroform with vigorous shaking, and the phage thus released are titered.

The time of dilution of the RNA–protoplast mixture with PAM medium is critical. The optimum time occurs from 30 to 45 seconds after addition of protoplasts to the RNA. The plaque titer drops very rapidly as longer periods of time are allowed.

The yield of phage is about one plaque-forming particle per 50 molecules of viral RNA input. This is a 100-fold improvement over the efficiency of infection obtained by application of the earlier method. The assay, using purified MS2 RNA as a standard, is linear over the range from about 10^6 to 5×10^{10} RNA molecules/ml. in the adsorption tube (0·8 ml. volume). Single burst experiments have indicated a burst size of about 2000 under these conditions; this would mean that we obtain one infected protoplast per 10^5 RNA molecules.

Unlike Ginoza & Vessey, we do not find bovine serum albumin to be inhibitory. Rather, it is desirable, and its omission lowers the efficiency of infection by one order of magnitude.

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† In Guthrie & Sinsheimer (1963) an error appears in the composition of PA and PAM media. These should contain 1 g glucose/l.

Some Characteristics of the Modified Assay

A growth curve of MS2 in RNA-infected protoplasts is shown in Figure II-2. By analogy with growth curves of phage-infected bacteria, the "eclipse period" is 25 to 30 minutes. Lysis of some of the infected protoplasts begins 5 to 10 minutes later; production of phage and lysis continue for about 2 hours. In this experiment the infected centers apparently rise between 5 and 20 minutes. This effect is not usually observed; the infected center count is normally constant between 5 and 25 or 30 minutes after infection.

It is not certain in the case of W6 protoplasts if the observed lysis occurs in the incubation tube or is produced in the plating procedure. Experiments with A19 protoplasts, which are infectible by the mature virus, show that in this case about one-third of the infected protoplasts lyse in the incubation tube. Growth curves of MS2 in RNA-infected A19 protoplasts do not begin to level off until after three hours of incubation; after this there is a slower rise in phage titer for at least an additional two hours. After five hours of incubation apparent burst sizes of 10^8 are produced. Thus some of the infected protoplasts lyse, leading to multiple infection cycles. An experiment which quantitates this fraction which lyse is shown in Table II-3. In this single burst experiment

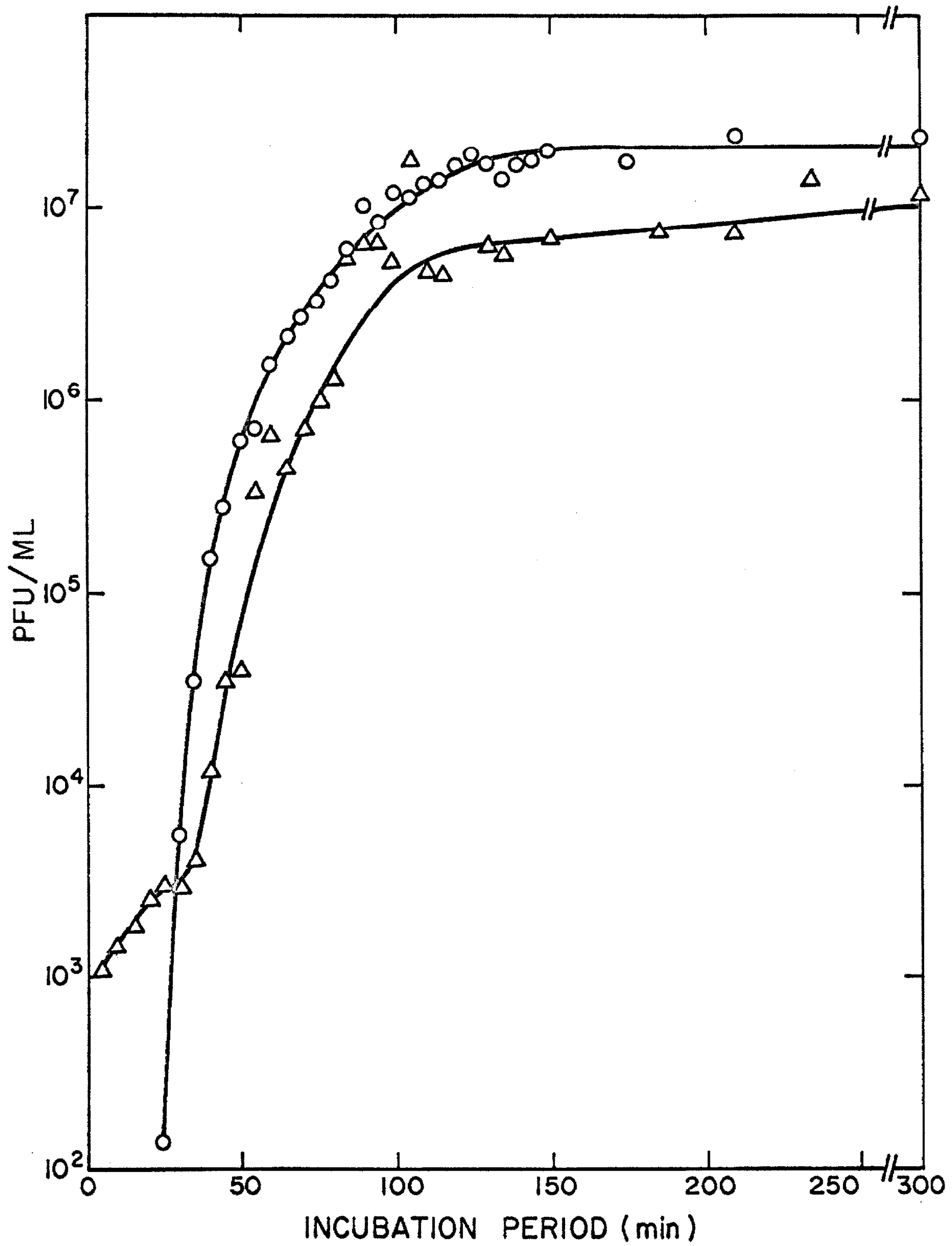

Fig. II - 2

Figure II-2 Growth Curve of MS2 in RNA-Infected Protoplasts

MS2-RNA at 5 x 10^{10}/ml. in 0.05 M tris pH 7.0 was placed in a number of tubes in a 37° bath. To each tube was added an equal volume of protoplast stock, and 30 sec. later the infection mixture was diluted five-fold with PAM medium. The tubes were then incubated at 37.0°C. From one tube aliquots were removed at intervals and infective centers plated. The other tubes were removed at intervals, the infected protoplasts lysed by the addition of $CHCl_3$, and assayed for phage titer. After 5 hours the infected protoplasts in the tube serving for assay of infective centers was lysed by $CHCl_3$ addition; the phage titer was found to be the same as that reached in the other tubes.

Δ-Δ Infective centers, not lysed

O-O Phage titer, protoplasts lysed by $CHCl_3$ addition

Table II - 3

Single Bursts in A19 Protoplasts

Number of Tubes	Average "Burst Size"	Range of "Burst Sizes"
75	0	--
17	1400	35 to 3810
8	9.4×10^8	1.6×10^6 to 6.0×10^9

A19 cells were converted to protoplasts with a 30 second conversion time. MS2-RNA was 2.5×10^6/ml. in 0.05 M tris pH 7. The infection time was 30 seconds, incubation was for 4 hours at 37°C.

Note that if we apply the zero order term of the Poisson distribution, $p_o = e^{-m}$, with $p_o = 0.75$, we have $m = 0.29$, i.e., the multiplicity of infection is 0.29. Since there are 0.4 ml. x 2.5×10^6 RNA/ml. = 1.0×10^6 RNA/tube, there are 0.29×10^{-6} infections per RNA molecule.

17 of 25 bursts gave burst sizes of 35 to 3800, reasonable figures for single bursts. The remaining 8 bursts all gave "burst sizes" of greater than 10^6; clearly these are due to lysis of the original infected protoplast followed by subsequent reinfection cycles.

We expect this partial lysis to hold for W6 protoplasts also. At any rate, lysis is never complete and must be completed artificially.

Plating for infected protoplasts is one-tenth to one-third as efficient as the assay based upon progeny phage. By the latter we mean the assay whereby the infected protoplasts are incubated for some time, lysed artificially, and the progeny phage titered. From the phage titer the number of infected protoplasts is calculated from the burst size measured in single burst experiments. In the case of W6 protoplasts the burst size is simply an average measured in several experiments. For A19, however, we have used a specific infectivity for the RNA calculated from a Poisson distribution (as outlined in the legend to Table II-3) together with a growth curve of MS2 in RNA-infected A19 protoplasts. The number of infected protoplasts calculated from the progeny phage titer is usually three-fold higher than those measured by plating for infected protoplasts, and at times is as much as ten-fold higher. Control experiments indicate that this is not due

to a lower efficiency of plating of the mature virus under the conditions for assay of infected protoplasts. Since the top agar used in this assay is PAM medium containing 0.8% agar, we suspect that this lowered efficiency is due to incomplete lysis of the infected protoplasts on the plate in analogy with the conditions in liquid medium.

A typical dilution curve for MS2-RNA is shown in Figure II-3. Duplicate points have been included to illustrate that the reproducibility of this assay is usually quite good. Phage production is linear with RNA concentration over at least four orders of magnitude; we have consistently observed that the assay is linear with RNA concentration.

With the better protoplast stocks, using saturating amounts of RNA, about 1% of the protoplasts can be infected.

The inhibition of the assay by TMV-RNA and *E. coli* ribosomal RNA is shown in Figure II-4. Inhibition begins when the competing RNA concentration is about 0.004 absorbancy units/ml. It will be noted that the dilution curve for MS2-RNA flattens out at an RNA concentration of 10^{11}/ml. (or 10^{10}/ml. upon dilution into the incubation tube); this is also an RNA concentration of 0.004 absorbancy units/ml. Thus our protoplast stocks are saturated by this concentration of RNA. With TMV-RNA the inhibition

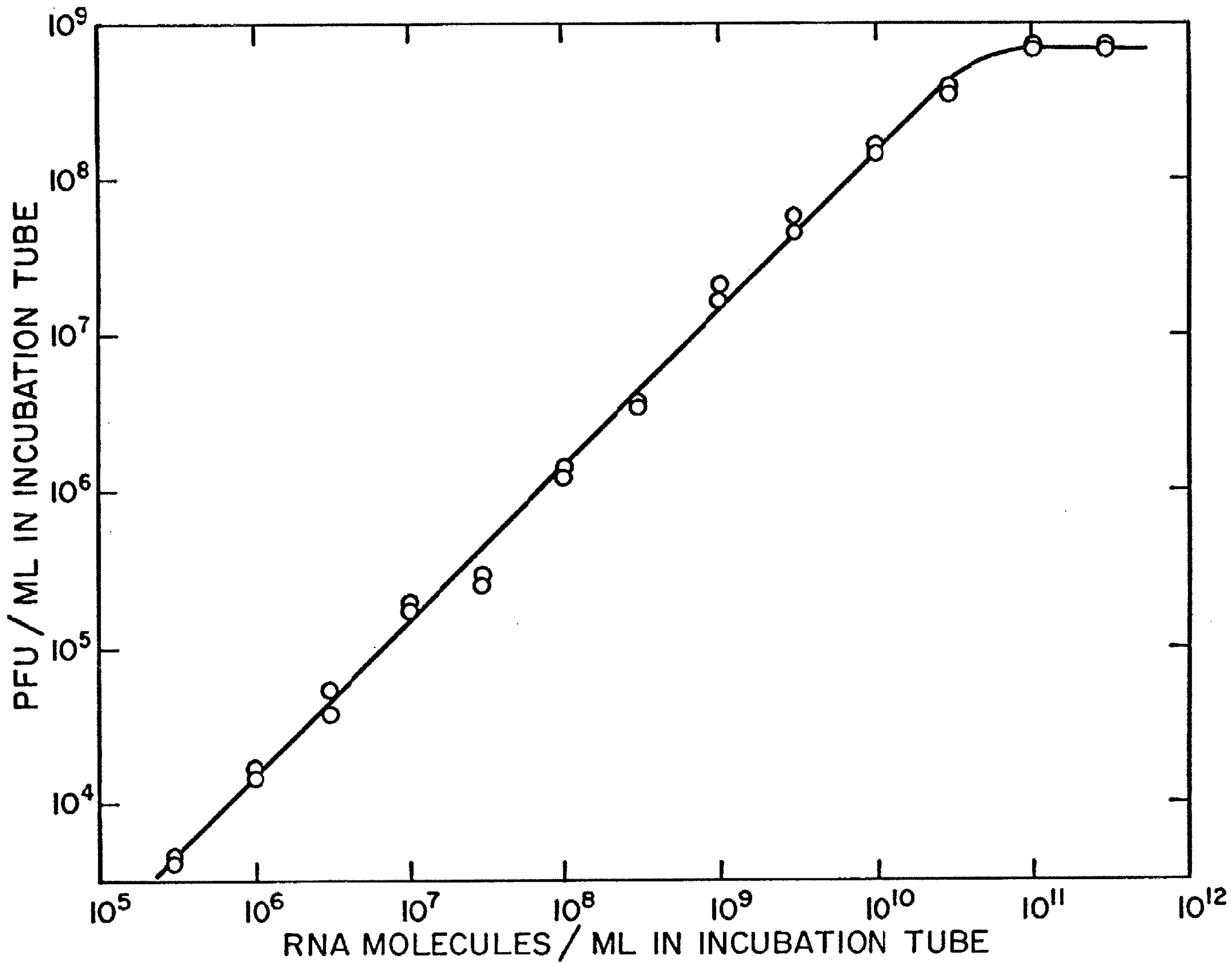

Fig. II - 3

Figure II-3 Dilution Curve of MS2-RNA

MS2-RNA at ten-fold the concentration shown in the figure, in 0.05 M tris pH 7.0, was mixed with an equal volume of protoplast stock, and 30 seconds later this mixture was diluted five-fold with PAM medium. Incubation followed for 90 minutes at 37°C. The line shown has a slope of 1.00 and gives 1.5×10^{-2} phage/RNA molecule.

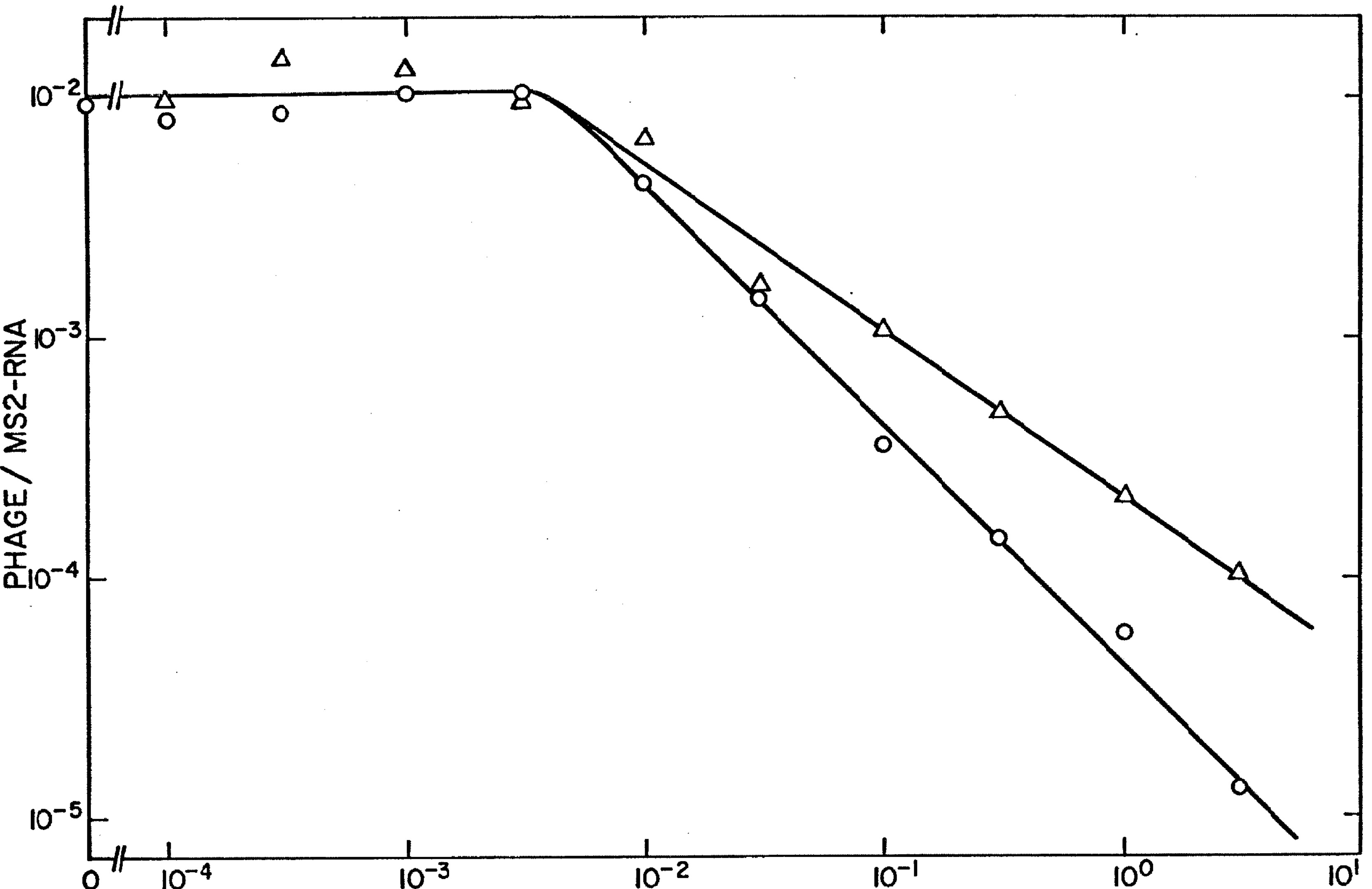

Fig. II - 4

Figure II-4 Inhibition of the Assay by TMV-RNA and Ribosomal RNA

TMV-RNA and *E. coli* ribosomal RNA were diluted to the concentrations shown in the figure in 0.05 M tris pH 7. MS2-RNA was added to a concentration of 10^{10} molecules/ml., and the mixed RNA's were assayed with an infection period of 30 seconds and an incubation period of 3 hours at 37°C.

O-O TMV-RNA

Δ-Δ *E. coli* ribosomal RNA

is then linear with increasing concentration, implying a strict competition by the RNA's for uptake or some other limiting factor. For E. coli ribosomal RNA, however, the inhibition goes as the 0.7 power of the concentration, implying that MS2-RNA may be preferentially taken up.

The Influence of External RNase on the Assay

The release of RNase I into the medium upon conversion of E. coli cells to protoplasts has been reported by Neu and Heppel (12,13). We have performed semiquantitative measurements on the amount of RNase in protoplast stocks and found the equivalent of about 15 ng/ml. of pancreatic RNase. Some of this is contributed by the BSA used. We have found different RNase activities in various lots of BSA used, but in general the RNase contamination is about 10^{-4}% (expressed as an equivalent weight contamination of pancreatic RNase). Thus the BSA contributes roughly one-third of the RNase activity in protoplast stocks.

We originally hypothesized that the low infectivity of RNA compared to DNA and the characteristics of the modified assay were a reflection of the presence of this nuclease. Under optimum conditions and using saturating amounts of RNA about 1% of the protoplasts can be infected; this is the same percentage as for ØX174-DNA (2). Thus the competence of protoplast stocks seems to be the same for DNA as for RNA. Short conversion times might release less nuclease and thus explain the increase in efficiency. Finally, the presence of an optimum in the time of infection (in contrast to DNA infection, ref. 2) could be explained by a competition between uptake of RNA by the protoplasts and RNA degradation. In this case, however, we must assume that most of the RNA is still susceptible to the external RNase for 10 to 20 minutes after a successful contact.

Experiments designed to test this hypothesis directly indicate that while RNase does affect the assay, it is not the limiting factor.

Protoplast stocks prepared from the strains A19 and Q13, which lack RNase I (11), have been investigated. For these strains, short conversion times (30 to 60 seconds) are also more effective. Thus the primary effect of short conversion times on RNA infectivity does not seem to be a reduction of RNase concentration. In addition, the

optimal time of infection is still 30 to 45 seconds.

The efficiency of A19 protoplasts is about 5 to 10-fold less than stocks of W6, despite the reduction in RNase present. In order to test whether the low efficiency was due to residual RNase contributed by the BSA we have prepared stocks using Armour 30% BSA treated with bentonite (14) (this treatment removes at least 99% of the RNase present).

The results of one such experiment are reported in Table II-4. This table also gives the results with the same stocks for ØX174-DNA infection. Although the mechanism of uptake and subsequent initiation of infection may be quite different for DNA and RNA, it was hoped that some insight into the infectivity assay might be gained from the comparison.

First it will be noted that the ratio of efficiency of the A19 protoplast stock to that of the W6 stock is roughly the same for DNA as for RNA. The absence of RNase I in the A19 stocks does not stimulate the RNA assay.

The use of bentonite-treated BSA in the protoplast stocks is inhibitory for DNA infection (10-60%); it is also inhibitory to the same extent in W6 stocks for RNA infection with a 30 second infection time, but provides some protection in a 10 minute infection. Bentonite treated BSA provides a slight stimulation with A19 stocks

Table II - 4

RNase and the Infectivity Assay

Nucleic Acid	pH	Infection Time	$\frac{A19}{W6}$	$\frac{A19(B)}{W6}$	$\frac{W6(B)}{W6}$	$\frac{W6(10)}{W6}$	W6
RNA	7.0	30"	0.13	0.16	0.88	0.22	1.3×10^{-6}
		10'	0.03	0.19	1.5	0.61	1.0×10^{-7}
	8.1	30"	0.17	0.23	0.39	0.20	1.5×10^{-6}
DNA	7.0	30"	0.23	0.15	0.51	0.35	7.8×10^{-5}
		10'	0.20	0.15	0.62	1.20	2.8×10^{-4}
	8.1	30"	0.23	0.16	0.30	0.24	2.1×10^{-4}
		10'	0.19	0.18	0.59	1.40	1.8×10^{-3}

Legend on following page.

Table II - 4 Legend.

The efficiency in stock W6 is given. For the other stocks the ratio of efficiency to that in the W6 stock under the same conditions of pH and time of infection is given.

Protoplast stocks were prepared from cells of W6 or A19. Stocks A19 and W6 were prepared with Armour 30% BSA and a conversion period of 30 seconds. Stocks A19(B) and W6(B) were prepared with Armour 30% BSA which had been treated with bentonite, and a conversion period of 30 seconds. Stock W6(10) had a conversion period of 10 minutes.

MS2-RNA at 10^{10}/ml. or ØX-DNA at 10^{8}/ml. in 0.05 M tris at either pH 7.0 or 8.1 was mixed with an equal volume of the protoplast stock. After 30 seconds or 10 minutes at 37°, the mixture was diluted five-fold with PAM medium. Incubation at 37° followed for 3 hours in the case of W6 protoplast infection or 2.0 hours in the case of A19.

Efficiencies were calculated from phage titer as follows. For ØX infection a burst size of 100 was assumed throughout. For MS2 a burst size of 2000 was assumed in W6 protoplasts. In A19 the figure used was 2.3×10^{5} phage/infected protoplast. This figure was based on a growth curve of MS2 in RNA-infected protoplasts in which infective centers before and after lysis were measured,

Table II - 4 Legend continued.

and in which the specific infectivity of the RNA was also measured from the Poisson distribution of bursts at low RNA concentration.

for RNA infection for both infection times. Note also that a 30 second infection period gives a better efficiency than a 10 minute infection.

These results indicate that external RNase does play some role in the RNA assay. Since large changes in RNase concentration have such little effect on the assay, however, it does not seem to be the limiting factor. Furthermore, removal of most of the RNase from the A19 stock has little effect on the decay of infected centers when the infection period is lengthened from 30 seconds to 10 minutes.

This decay of infected centers, which gives rise to an optimum in the time of infection, thus seems not to be caused by external RNase. Presumably, then, some other step in the uptake or infection process can be undertaken, at least efficiently, only upon dilution of the infection mixture with PAM medium, and if this step is not performed within a limited time interval the infected center is lost.

The optimum in the infection period provides a partial explanation for the lowered efficiency of RNA compared with DNA. In the experiment reported in Table II-3 only about 10% (pH 7) or 25% (pH 8.1) of the total DNA-infected protoplasts achieved were formed in the first 30 seconds of infection. It is perhaps of significance also that for DNA infection W6 protoplasts prepared with a short

conversion time are more efficient for a 30 second infection, but less efficient for a 10 minute infection.

DISCUSSION

The efficiency of the modified assay in our hands has varied from about 10^{-5} to 10^{-6} infections per RNA molecule (assuming a burst size of 2000). If care is taken, the efficiency is normally the higher value. Glowacki (personal communication) has obtained this efficiency with both MS2-RNA and Qβ-RNA. Other investigators (15, 16) have had difficulty reproducing this efficiency. Part of this variation may be related to the BSA solution used. Since we have found that Fraction V BSA will not substitute for the Armour 30% solution, there is evidently some contaminant in this solution which is at least partially responsible for the efficiencies obtained.

Recently Franke and Hofschneider (17) have quoted an as yet unpublished assay which gives efficiencies comparable to this assay.

The results on ionic strength of the RNA solvent have not been confirmed by Glowacki (personal communication), who found a distinct optimum of 0.05 M tris for pH 6.2 to 6.9. The variation in efficiency at 0.05 M or below is again of the order of a factor of two, so that this effect is not critical. Her results in phosphate buffer

were similar to ours and at the respective optima gave the same efficiency as tris buffers.

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PART III

THE INITIAL KINETICS OF DEGRADATION OF MS2-RNA BY RIBONUCLEASE, HEAT, AND ALKALI

INTRODUCTION

The experiments reported in this section were performed as controls for the experiments in Part V. The kinetics of RNase degradation were studied so as to be able to deliver a predetermined number of hits to MS2-RNA samples. Inactivation at high pH was studied for this purpose and for its possible use as a denaturing agent. The stability of the RNA at elevated temperatures was examined to determine the amount of heating to which the RNA could be subjected without degradation. The results seem sufficiently interesting to warrant separate presentation.

MATERIALS AND METHODS

The RNA concentration is expressed in molecules/ml. as in Part II. The concentration of pancreatic RNase is also expressed in molecules/ml., calculated from the weight concentration and the conversion factor of 4.4×10^{16} RNase molecules/mg.

Bovine pancreatic RNase is Sigma IA, five times crystallized. Several lots have been used. These lots were quoted by Sigma as being either 42 or 60 KU/mg, and corresponding differences in activity were found. All lots quoted as 60 KU/mg, however, gave the same activity toward MS2-RNA.

RNase was diluted through 0.02% BSA for most assays. The diluted RNase solution was mixed with an equal volume of RNA in the desired buffer, and the mixture placed at 37°C. Aliquots were removed at intervals and diluted into ice cold 0.05 M tris pH 7, then further diluted if necessary. From the final dilution 0.4 ml. aliquots were placed in tubes and frozen immediately at dry ice temperature. Samples were stored frozen at -15°C until a few minutes before assay (within one or two days).

Alkali inactivations were performed by mixing a solution of RNA with either phosphate buffers of the desired pH, or with NaOH solutions. In the latter case the RNA was in 0.1 M phosphate pH 7.0 (0.04 M NaH_2PO_4 -0.06 M Na_2HPO_4), the NaOH was 0.14 N. The pH was determined from a calibration curve of pH versus volume of NaOH added. pH readings were made with a Corning blue glass electrode and are given without correction. All inactivations were performed at 0°C. Aliquots were removed at intervals and diluted into 0.05 M tris pH 7 for assay.

Thermal inactivations were done by heating the RNA in the desired buffer to the desired temperature; aliquots were removed at intervals and diluted into 0.05 M tris pH 7 for infectivity assay.

Infectivity assays were performed by one of the

methods discussed in Part II.

In all cases the rate constant for inactivation, k, was found from the zero order term of the Poisson distribution: $P_o = \exp(-kt)$ where P_o is the surviving infectivity at time t. Where time course studies were performed, k was evaluated from a semilogarithmic plot of P_o versus t. This rate constant can also be expressed as the mean number of bonds cleaved per molecule of RNA (m) per unit time: $k = m/t$. m will be referred to as the number of (biological) hits. In the case of RNase the results are expressed as a turnover number, the number of bonds cleaved/min./RNase molecule; or, turnover number = k (RNA/RNase) where both RNA and RNase are in molecules/ml.

RESULTS AND DISCUSSION

RNase Inactivation

The turnover number of RNase is almost independent of RNA and RNase concentration within the limits tested. For RNA these limits were 10^{11} to 10^{15} molecules/ml. Lower concentrations were not tested because RNase contamination of the BSA becomes limiting, and in the absence of BSA the RNase may be slowly inactivated, losing most of its activity in 20 to 30 minutes. For RNase the concentration limits are that 0.5 to 5 hits be delivered to the RNA in an hour. Table III-1 illustrates this result.

Table III - 1

The Turnover Number of Pancreatic RNase

RNase molecules/ml.	RNA molecules per ml.						
	2×10^{11}	7×10^{11}	2×10^{12}	7×10^{12}	2×10^{13}	7×10^{13}	2×10^{14}
2.2×10^{7}	510						
7.4×10^{7}	360	310					
2.2×10^{8}		310	560				
7.4×10^{8}			550	650			
2.2×10^{9}				540	810		
7.4×10^{9}					600	1300	
2.2×10^{10}						1050	1300
7.4×10^{10}							1000

RNA and RNase at the indicated concentrations were incubated at 36°C in 0.05 M tris pH 7 plus 100 γ/ml. BSA for 10 minutes. The samples were then diluted at least ten-fold into 0.05 M tris to give an RNA concentration less than 4×10^{10}/ml. and the infectivity assayed.

The data in Table III-1 appear to show a small dependence on concentration. It is not certain whether this effect is real or is experimental error. When the turnover number is more carefully determined from time course studies we usually find it to be independent of concentrations within the limits stated. At any rate, a change of three orders of magnitude in RNA and RNase concentrations produces at most a change of a factor of two in the turnover number of RNase.

The data in Table III-1 are based on a single time point, 10 minutes. When the inactivation is followed as a function of time it is found to be exponential for at least 60 minutes or two log cycles when the RNase is protected with BSA. Figure III-1 shows such inactivation curves.

The results of such inactivation curves have always given a turnover number dependent only upon the temperature and buffer system used. This makes RNase a convenient reagent for the purposes of Part V. The turnover numbers at $37^{\circ}C$ in three buffers are given in Table III-2: these refer to RNase lots quoted by Sigma as 60 KU/mg.

The turnover number at $0^{\circ}C$ in 0.05 M tris (pH 7 at 25°) is one-tenth that at 37°

Inactivation rates in organic solvents were studied

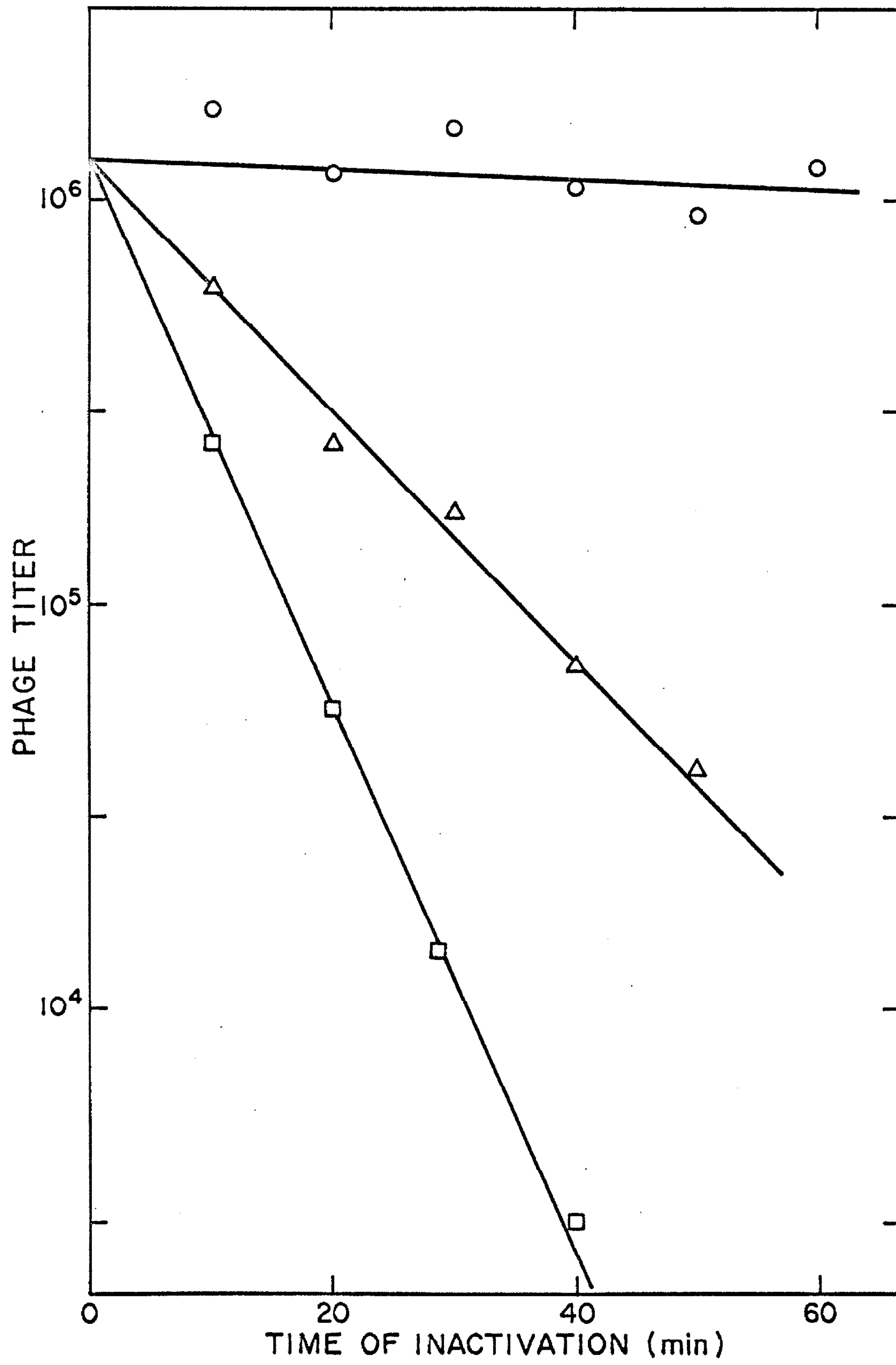

Fig. III-1

Figure III-1 The Inactivation of MS2-RNA with RNase

The inactivations were conducted at 37°C in 0.05 M tris pH 7 plus 100 γ/ml. BSA. The RNA concentration was 6.0×10^{14}/ml. At the indicated times aliquots were removed and diluted to an RNA concentration of 2.0×10^{10} for infectivity assay.

O - O No RNase

Δ - Δ 2.2×10^{10} RNase molecules/ml.

□ - □ 4.4×10^{10} RNase molecules/ml.

Table III - 2

Effect of the Solvent on the Turnover Number of RNase at 37^oC.

Solvent	Turnover Number
0.05 M tris, pH 7 (at 4^oC)	640
0.05 M tris, pH 7 (at 25^oC)	1800
0.05 M phosphate, pH 7	15000

Table III - 3

Inactivation of MS2-RNA at High pH

pH	μ	k (min.$^{-1}$)	k/$[OH^-]$	$k_o/[OH^-]$
10.95	0.3	0.0033	3.7	1.0
11.85	0.3	0.031	4.3	1.2
12.27	0.3	0.106	5.4	1.6
12.22	0.6	0.18	10.3	1.7

The rate constants were determined from time course inactivations at 0^oC at the pH shown. μ is the ionic strength, k_o is determined from the relation log $(k/k_o) = \sqrt{\mu}$.

to some extent. At concentrations of dimethylsulfoxide or formamide of 80% or greater the inactivation rate is much slower and no inactivation is detectable after 10 minutes at 37°C. In 50% formamide inactivation is significant and at 0°C is faster than in the corresponding aqueous solvent.

In Part V it is shown that for RNase inactivation the biological hits correspond closely to the number of hits measured by analytical centrifugation: each biological hit corresponds to chain cleavage. Similar results have been found for TMV-RNA by Gierer (1, 2).

Inactivation at High pH

The inactivation of MS2-RNA by alkali at 0°C is exponential for at least two log cycles. At pH's of 12 or greater we have found a biphasic curve: the slope for the first minute is greater. This is probably due to thermal differences caused by pipetting together of the solutions and removal of aliquots. Inactivation curves at two pH's are shown in Figure III-2.

The rates of inactivation at several pH's are listed in Table III-3. Also shown in the table are the rate constants corrected for hydroxyl ion concentration and for ionic strength. The rate is almost proportional to the hydroxyl ion concentration over the 20-fold range tested, and the ionic strength dependence is in agreement with the

Brönsted relation: $\log (k/k_0) = 2AZ_1Z_2$ where $2A = -Z_1 = -Z_2 = 1$. Similar results have been found for yeast RNA by Bacher and Kauzmann (3) using titrimetric data.

Thermal Inactivation

The inactivation of MS2-RNA at three temperatures was followed. At each temperature the inactivation was exponential. The data for two temperatures are shown in Figure III-3. In Figure III-4 is shown an Arrhenius plot of the data. The activation energy is 22 kcal./mole. This may be compared with activation energies of 19 kcal./mole for TMV-RNA found from inactivation of infectivity (4) or from measurements of the decrease in molecular weight (5), and 27 kcal./mole for yeast RNA from titrimetric studies (3).

Our absolute thermal rate constants (rate per phosphodiester bond) are two to three times higher than those found for TMV-RNA or R17-RNA when the degradation is followed by physical measurements of chain cleavage (5, 6). They are one-half that found for TMV-RNA from inactivation of infectivity (4). Thus it is likely that only a fraction of the infectivity hits, 30 to 50% for MS2-RNA, are due to thermally induced phosphodiester bond cleavage. The remaining fraction may be caused in whole or in part by depurination. If RNA depurinates at the same rate as single-stranded DNA under these conditions

(7), and if such depurination is lethal (8), about one-third of the biological hits would be due to depurination.

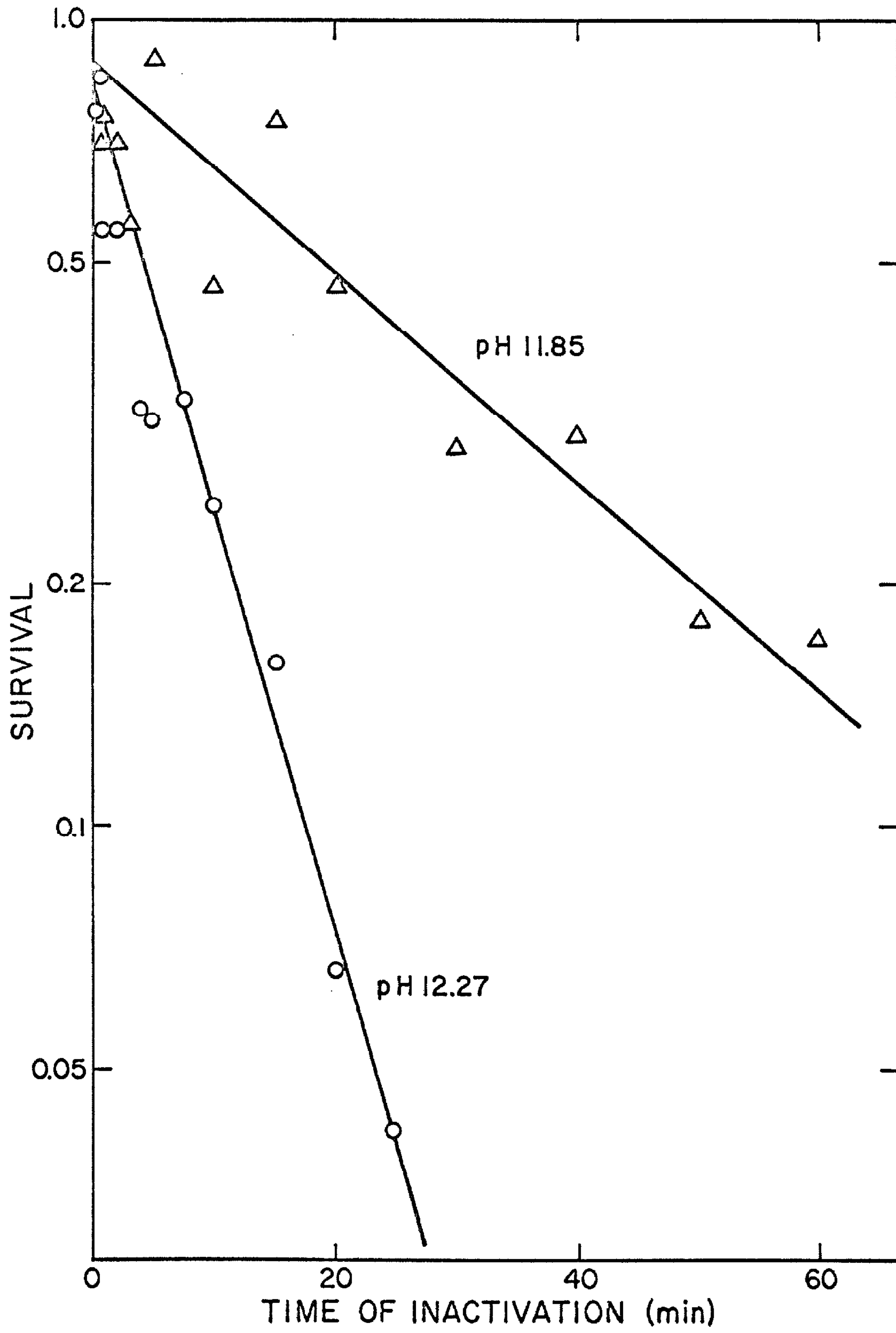

Fig. III - 2

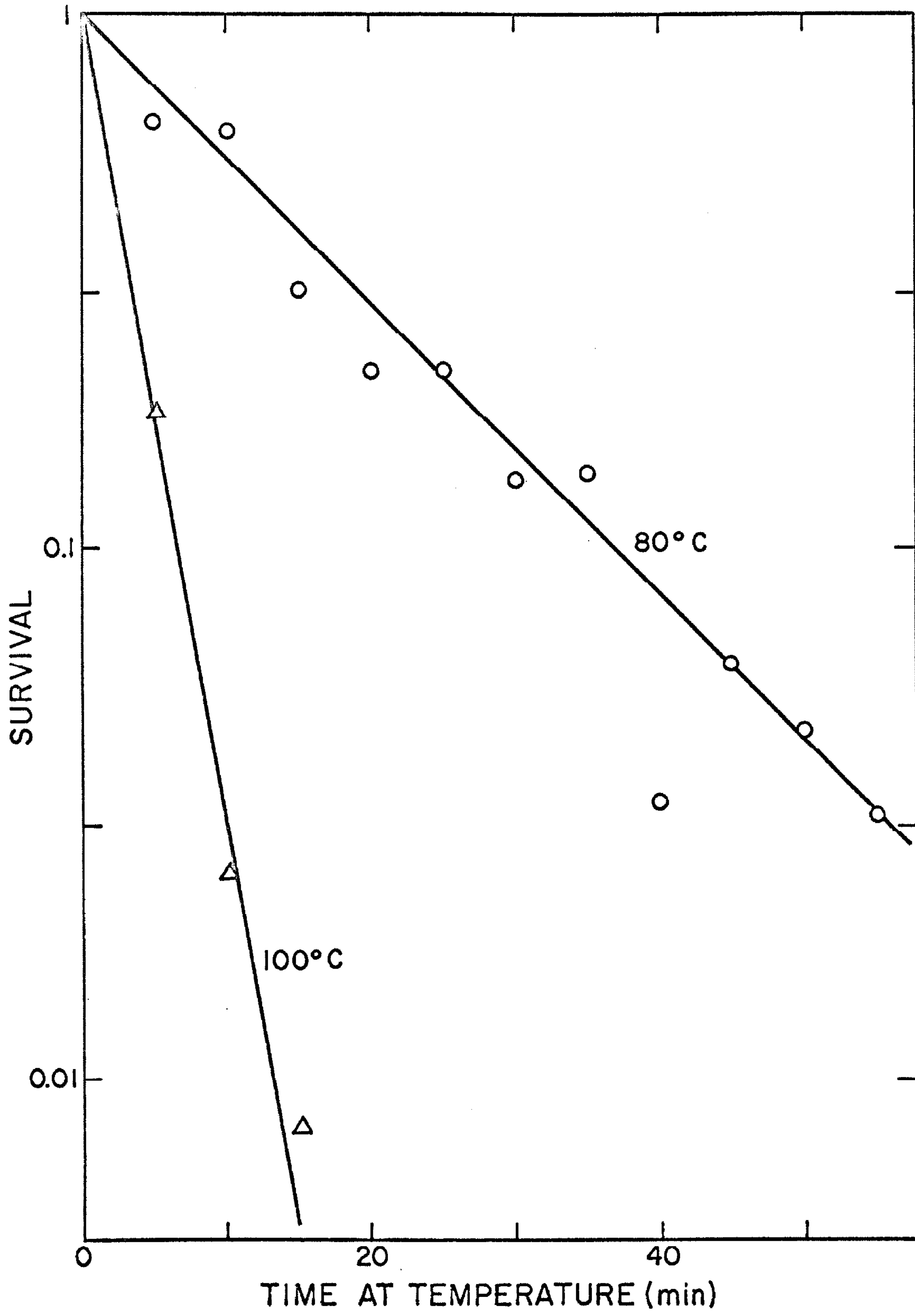

Fig. III - 3

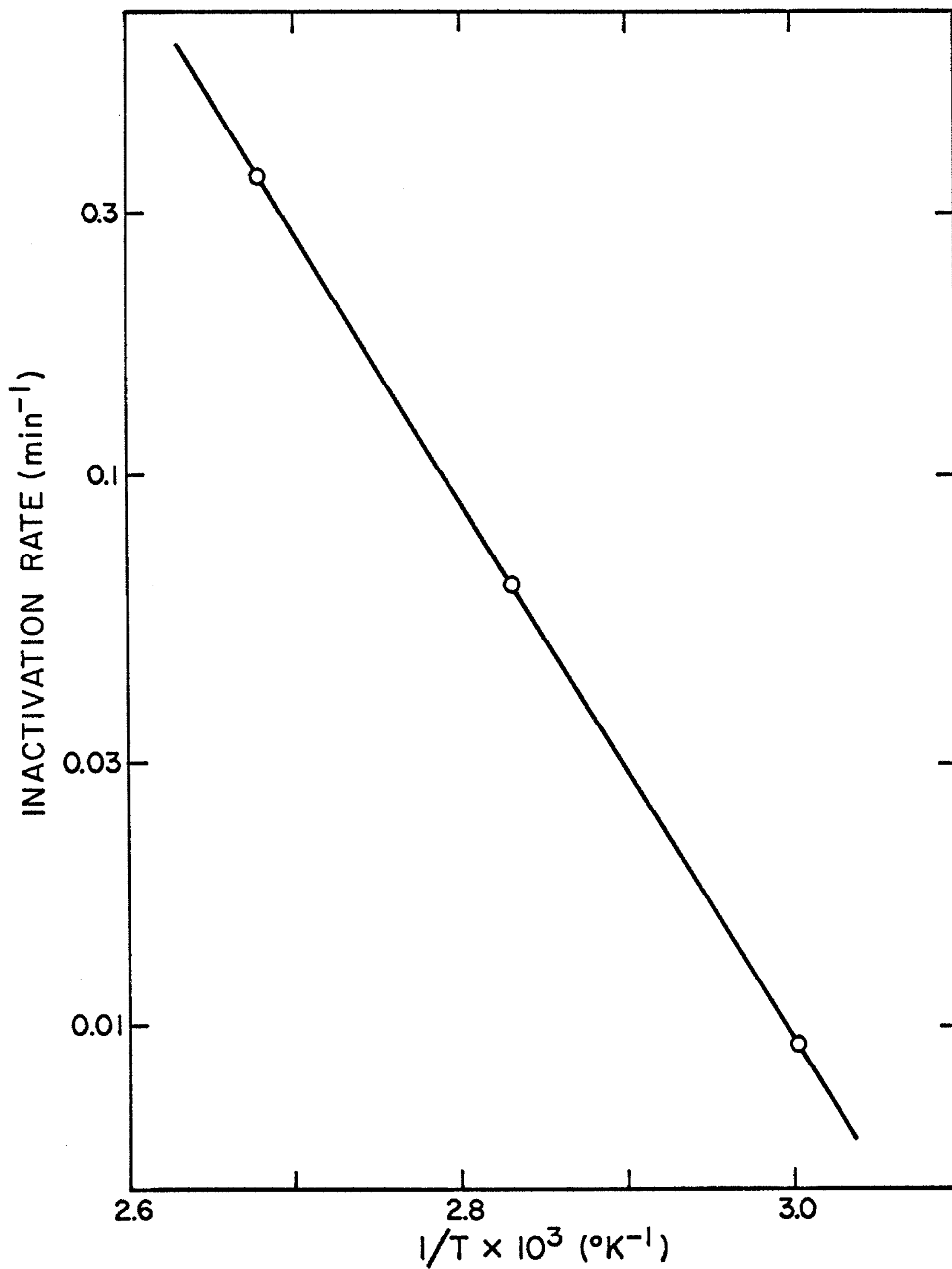

Fig. III - 4

Figure III-2 Inactivation of MS2-RNA at High pH

The RNA in 0.1 M phosphate was mixed with 0.14 M NaOH to the desired pH at 0°C. At intervals aliquots were removed and diluted 100-fold for infectivity assay. A control sample was stable for several hours.

O - O pH 12.27. RNA concentration for inactivation $= 1.9 \times 10^{12}$

Δ - Δ pH 11.85. RNA concentration for inactivation $= 2.4 \times 10^{12}$

Figure III-3 Thermal Inactivation of MS2-RNA

O - O 80°C, RNA at 3.7×10^{13}/ml. in 0.01 M phosphate pH 7

Δ-Δ 100°C, RNA at 2×10^{13}/ml. in 0.1 M phosphate buffer pH 7. The points shown are the average of two different inactivation curves.

Figure III-4 Arrhenius Plot of Thermal Inactivation Data

The inactivations at 60°C and 80°C were done in 0.01 M phosphate pH 7, that at 100°C in 0.1 M phosphate pH 7.

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PART IV

THE DENATURATION OF MS2-RNA AND OF DOUBLE-STRANDED FORMS OF RNA

INTRODUCTION

A large body of literature exists concerning the denaturation of DNA, in particular double-stranded DNA, and of model polydeoxyribonucleotides. Comparatively little is known for RNA.

The thermal and formaldehyde denaturation of (single-stranded) RNA was first characterized by Doty _et_ _al_., following the denaturation of TMV-RNA by ultraviolet hyperchromicity and optical rotation. The thermal denaturation of TMV-RNA was further explored by Boedtker (2). Viscosity measurements have been used by Spirin (3,4) to follow the thermal denaturation of TMV-RNA and the ribosomal RNA's from _E_. _coli_, pea, rabbit, and rat; the T_m of these RNA's was found to be linearly related to their GC content.

The thermal denaturation of soluble RNA's has been of some interest because of the partially double-stranded nature of these molecules; results obtained before 1964 have been reviewed by Brown (5). Fasman _et_ _al_. (6) have characterized the optical rotation properties of yeast soluble RNA and of the formaldehyde denatured product.

Helmkamp and Ts'o (7) have studied the denaturation of ribosomal RNA in the organic solvents dimethylsulfoxide and formamide.

We have felt that a study of the denaturation

conditions of double-stranded RNA as well as single-stranded was important, for if double-stranded RNA can be shown to be denatured under certain conditions we have confidence that no comparatively short double-stranded regions remain in a single-stranded RNA. Due to the lack of double-stranded RNA's and of model compounds until recently, few such studies have been made. Ammann et al. (8) have studied the ultraviolet transition and the formation of infective RNA upon thermal denaturation of a double-stranded form of phage M12-RNA. Haselkorn and Fox (9) have synthesized rG:rC and characterized some of its properties. Recently the denaturation of a double-stranded form of poliovirus RNA with dimethylsulfoxide has been reported (10).

Thermal denaturation of double-stranded RNA is difficult and impractical, for such RNA's have very high denaturation temperatures and rG:rC is almost impossible to melt by thermal treatment (9). Furthermore, the use of alkali (11) is ruled out with RNA because of the concomitant hydrolysis. Thus we have turned principally to organic solvents as a convenient method of denaturation.

MATERIALS AND METHODS

Absorption Spectra

Ultraviolet spectra were recorded with a Beckman DK2 Recording Spectrophotometer equipped with jacketed cells. Hyperchromicities were corrected for thermal expansion of the solvent. The data for aqueous solutions were taken from the Chemical Rubber Company Handbook. The data for DMSO and FA are shown in Figure IV-1. In solutions of intermediate DMSO concentrations the volume expansion factor was assumed to be the weighted average of that for water and DMSO.

The results have been expressed as a percent hyperchromicity, defined as % Hyperchromicity = $100\ (A-A_{ref})/A_{ref}$ where A is the absorbancy under the conditions of interest, and A_{ref} is the absorbancy of the same concentration of nucleic acid in SSC at 20°C at the same wavelength.

The extinction coefficients at 260 mμ used for the nucleotides were those of Beaven _et al_. (12), that of poly rG:rC was from Haselkorn and Fox (9).

Organic Solvents

DMSO was Baker reagent grade (99.9%). Different lots contained varying amounts of ultraviolet absorption. The optical density at 310 mμ was 0.1 to 0.6, and rose slowly with decreasing wavelength until about 275 mμ; the optical

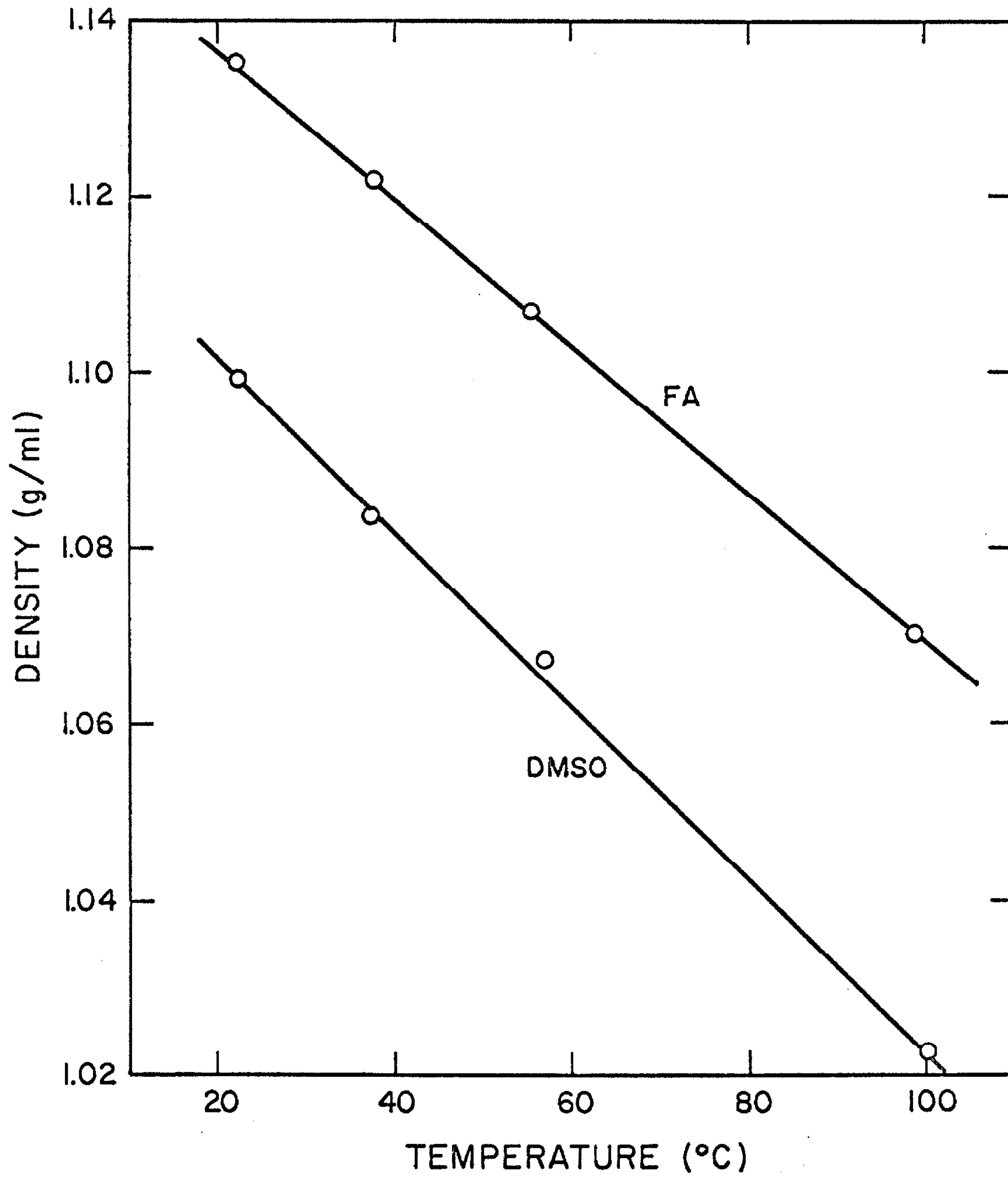

Fig. IV-1

Figure IV-1 The Density of DMSO and of FA as a Function of the Temperature.

The volume of a 10 ml. volumetric flask was measured at each temperature using distilled water as a standard. The densities of FA and DMSO were then measured at each temperature.

density rose rapidly in the 265 to 255 mμ region. Various lots were tested, and only those used which had an optical density of less than 0.3 with a one cm. pathlength at a wavelength of 275 mμ.

DMSO solutions were used with 0.001 M sodium EDTA pH 7.1. This concentration is saturating, and when it was desirable to increase the salt concentration LiCl was used.

Formamide is from Matheson, Coleman, and Bell, 99%. Before use it was tested to be neutral to brom thymol blue (13).

Preparation of su-11-RNA

An ethanol precipitated phenol extract of a lysate of non-permissive cells infected in the presence of C^{14}-uracil with su-11, a suppressor-sensitive mutant of phage f2 (14), was kindly supplied by Dr. R. C. Valentine. This material was treated with 25 γ/ml. of RNase and 10 γ/ml. of DNase for 45 minutes at 37°C in the presence of 0.01 M $MgCl_2$ and 0.2 M NaCl. The digest was phenol extracted four times, then ethanol precipitated five times. It was dialyzed against a large excess of 0.2 M NaCl containing 0.01 M tris and 0.01 M sodium EDTA pH 7.1. Then followed successive dialyses against 0.01 M tris plus 0.001 M sodium EDTA containing, in order, 0.2 M NaCl, 2M NaCl (twice), 0.2 M NaCl. The preparation was then dialyzed into 0.1 M tris pH 7 and stored at -70°C.

The final preparation was heterogeneous, with sedimentation coefficients from 0 to 13 S (as analyzed by either radioactivity or optical density). It was completely resistant to 10 γ/ml. of RNase for 30 minutes in SSC at 22°C, but 21% of the counts became soluble in trichloroacetic acid after this RNase treatment at 37°C. In SSC/10 97% of the counts became acid soluble after treatment with 10 γ/ml. of RNase for 30 minutes at either 22° or 37°C.

rG:rC

rG:rC was a generous gift of Dr. R. Haselkorn, and its properties are described by Haselkorn and Fox (9). The sample was dialyzed into 0.005 M LiCl containing 0.001 M sodium EDTA pH 7.1, then placed *in vacuo* over P_2O_5 at room temperature until the volume was reduced to about 15% of the initial volume.

Abbreviations used

DMSO = dimethylsulfoxide

FA = formamide

SSC = standard saline-citrate, 0.15 M NaCl plus 0.015 M sodium citrate pH 7.0

SSC/10 = 0.015 M NaCl plus 0.0015 M sodium citrate pH 7.0

ORD = optical rotatory dispersion

T_m = the midpoint of the thermal hyperchromicity

transition

rG:rC = polyriboguanylic acid complexed with polyribocytidylic acid

RESULTS

Thermal Denaturation

The thermal denaturation curve of MS2-RNA in SSC is virtually identical to the one shown in Part I in 0.14 M NaCl, with a displacement of the temperature axis by 13°. As noted in Part I, the T_m for preparations of MS2-RNA isolated from virus not exposed to EDTA was 76° in 0.15 M salt. Exposure of the virus to 0.01 M EDTA reduced this T_m to 63°. Addition of 0.001 M Mg^{++} to such an RNA preparation increases the T_m to 67°, whereas 0.01 M Mg^{++} brings the T_m to 76°. Whether the virus has been exposed to EDTA or not, the T_m in 0.02 M salt is the same, however.

The thermal denaturation of su-11-RNA in SSC and in SSC/10 is shown in Figure IV-2. Denaturation in SSC is incomplete at 100°, although a reasonable estimate of the T_m can be obtained. The T_m's and percent hyperchromicities at 100° are given in Table IV-1 for MS2-RNA and for su-11-RNA.

The melting curve for su-11-RNA shows a gradual rise in hyperchromicity with increasing temperature, followed by a sharp rise at elevated temperatures. The latter is characteristic of double-stranded RNA in that this T_m is quite high and in that this transition is much sharper than the single-stranded MS2-RNA transition. It is, however, not as sharp as might be expected (e.g., see ref.

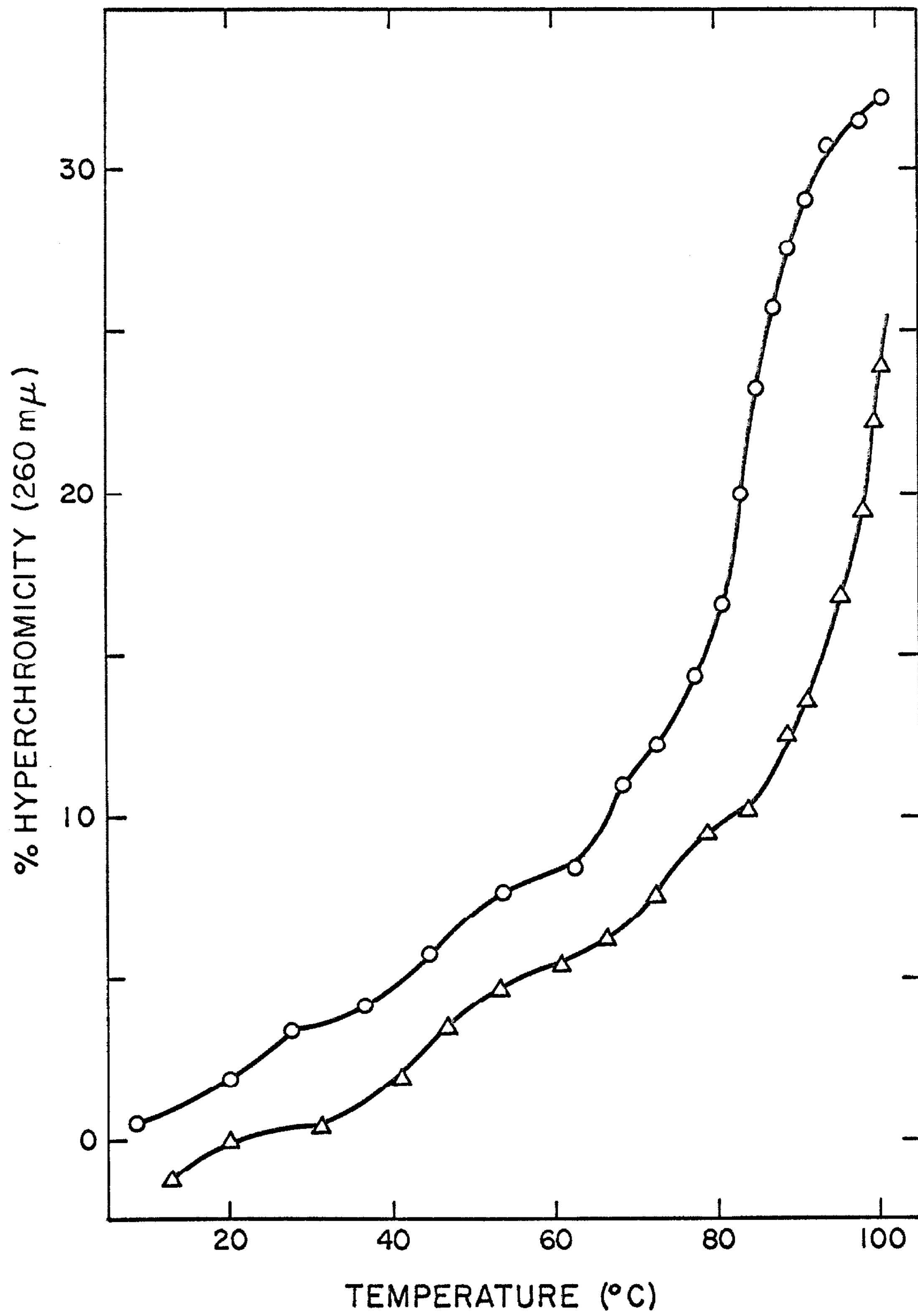

Fig. IV - 2

Figure IV-2 Thermal Denaturation of su-11-RNA

The absorbancy of su-11-RNA was followed as a function of the temperature. The results are expressed as percent hyperchromicity, defined in Materials and Methods.

Δ-Δ SSC

O-O SSC/10

Table IV - 1

T_m and Hyperchromicity Data

	MS2-RNA		su-11-RNA	
	SSC	SSC/10	SSC	SSC/10
T_m (260 mμ)	63°	49°	99°	84°
% Hyperchromicity at 100° (260 mμ)	33%	32%	(24%)	32%
T_m (280 mμ)	67°		99°	83°
% Hyperchromicity at 100° (280 mμ)	42%	40%	(31%)	44%

The RNA samples were diluted into the indicated solvent and the absorbancy followed as a function of the temperature. T_m is the midpoint of the thermal transition. Percent hyperchromicity is defined in Materials and Methods.

8). At least part of this broadening of the thermal transition is due to the heterogeneity of the preparation (e.g., ref. 15).

We interpret the fairly sharp increase of hyperchromicity occurring at elevated temperatures, accounting for 70-80% of the total hyperchromicity observed, as further evidence that the major fraction of this RNA preparation consists of double-stranded RNA. The remaining 20-30% of the preparation may consist of (single-stranded) purine tracts or very short stretches of double-stranded RNA. The latter may include longer double-stranded molecules in which one or both of the chains is interrupted at intervals. Lipsett et al. (15) have found that if only one of the polynucleotide strands is interrupted at short intervals the T_m is greatly reduced, and RNase probably attacks double-stranded RNA to some extent (9).

The T_m's for su-11-RNA shown in Table IV-1 are for the sharp transitions. This transition is sharp enough, however, that the T_m for the entire preparation differs from this by only about 3^oC.

The high temperatures necessary to denature su-11-RNA, taken together with the thermal inactivation rate constants of Part III, illustrate the difficulties of thermal denaturation of double-stranded RNA.

Formaldehyde Denaturation

The hyperchromicities at 25° exhibited by MS2-RNA after reaction with 1.8% formaldehyde in 0.01 M phosphate pH 7 for various lengths of time at several temperatures are given in Table IV-2. These data are included to demonstrate that the conditions chosen in Part V for formaldehyde denaturation of MS2-RNA lead to complete reaction with this reagent.

Denaturation in Dimethylsulfoxide

The hyperchromicities of MS2-RNA and of su-11-RNA as a function of temperature at several DMSO concentrations are given in Figures IV-3 and IV-4, respectively. These data are replotted to give the hyperchromicity at 23°C as a function of the DMSO concentration in Figure IV-5, and to give the T_m versus DMSO concentration in Figure IV-6. The T_m's for su-11-RNA again refer to the sharp transition. All solutions contain 0.001 M EDTA as the sole buffer.

As can be seen from Figures IV-5 and IV-6, increasing concentrations of DMSO have little effect on the hyperchromicity shown at 23°C or on the T_m until a critical concentration is reached. Then these properties change rapidly over a fairly narrow concentration range. The concentration of DMSO for "half-melting" of MS2-RNA at 23°C is 50% (v/v), whereas it is 70% for double-stranded su-11-RNA. MS2-RNA is completely melted in 60%

Table IV - 2

Formaldehyde Denaturation of MS2-RNA

Time of Reaction	Hyperchromicity at 25° after Reaction			
(min.)	100°C	90°C	80°C	60°C
1	33.9	26.5	27.2	
2	34.8	32.5		
3	36.1	33.9	34.1	28.0
5	35.7	35.9	35.4	
10	35.2	36.1	35.4	35.2
15			36.0	36.6
20	37.4			
30		36.6	37.3	37.7

MS2-RNA at 0.37 A_{260} in 0.01 M phosphate pH 7 containing 1.8% formaldehyde was heated to the indicated temperatures for the indicated times. The samples were then cooled and the absorbancies read at 25°C.

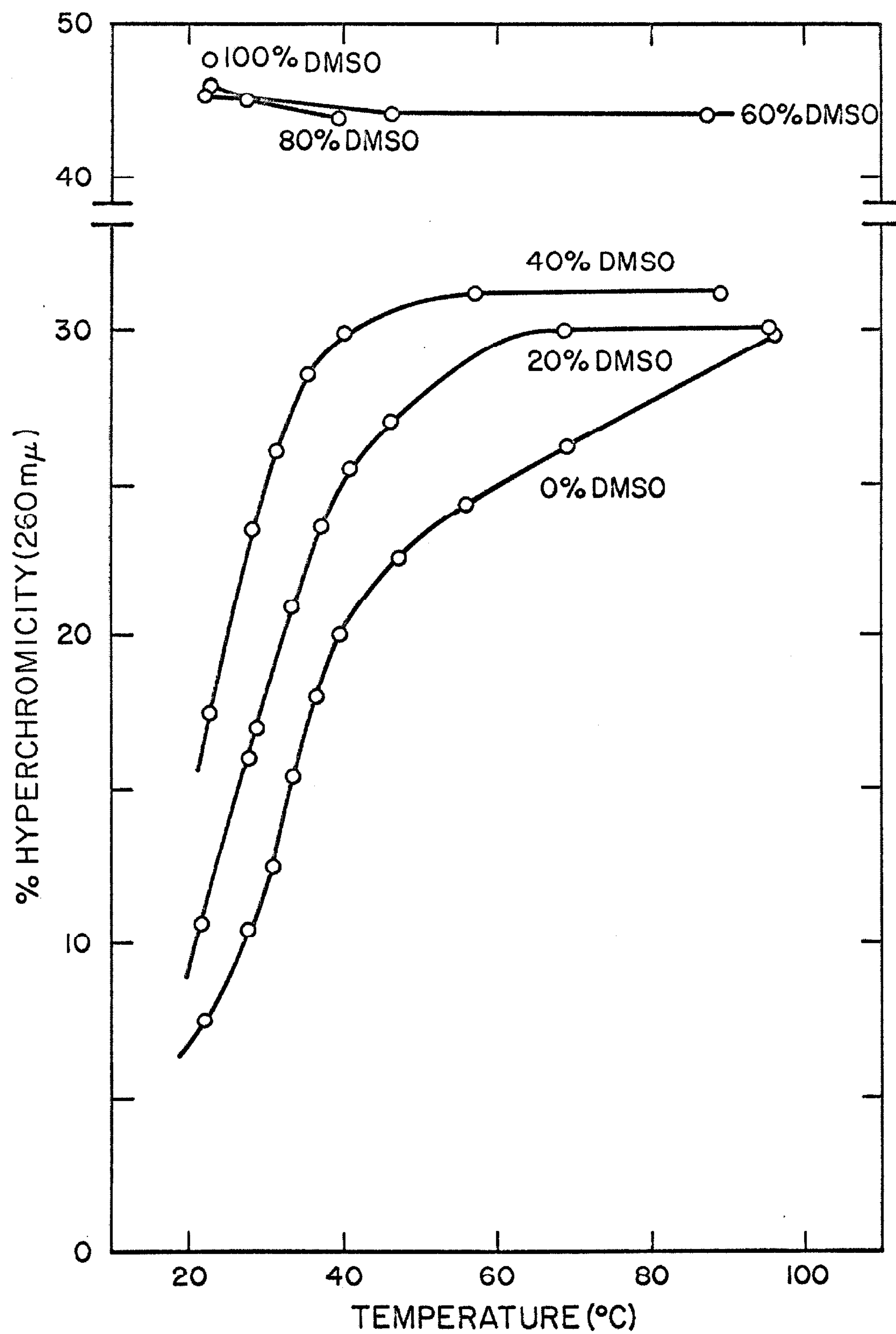

Fig. IV-3

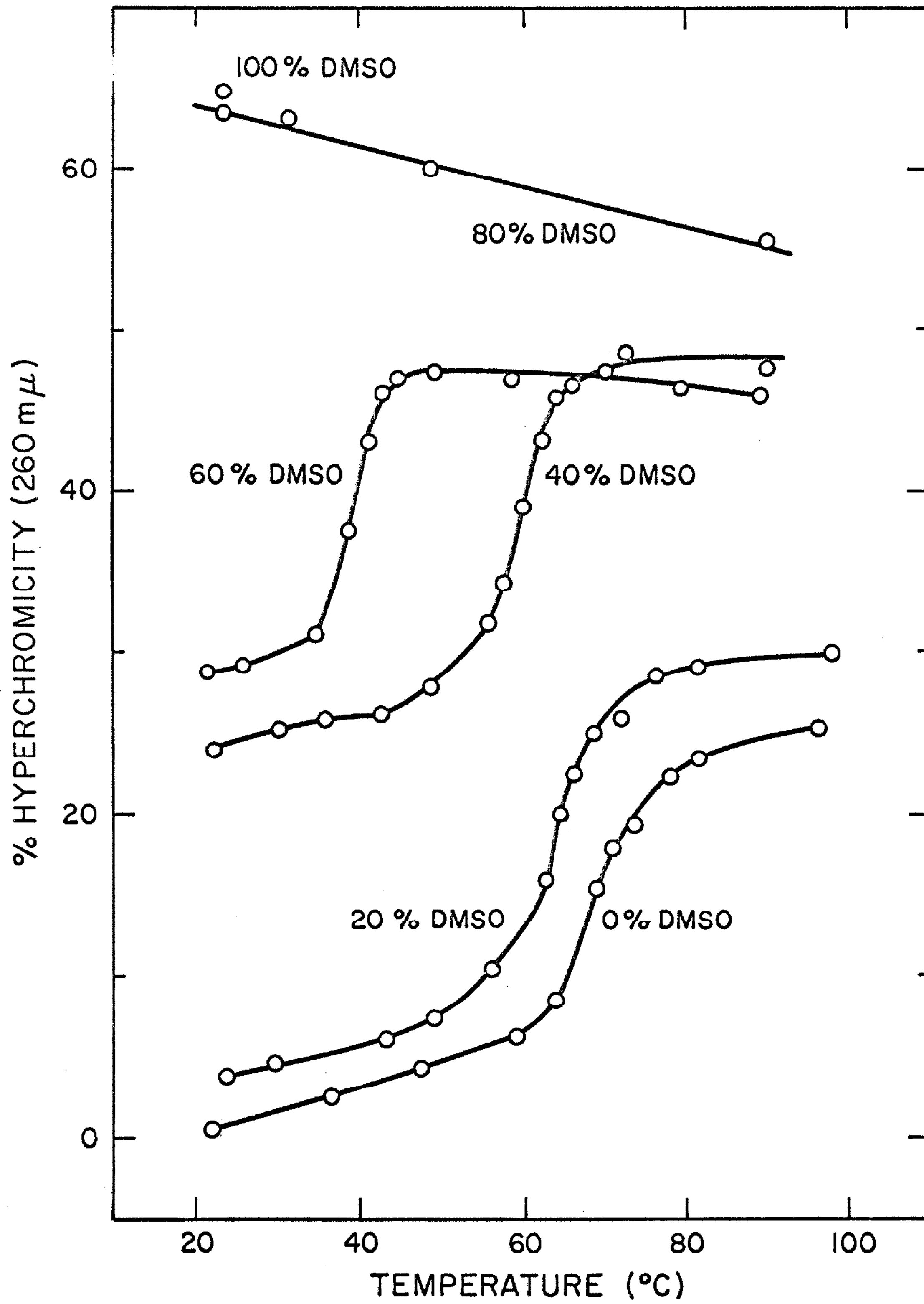

Fig. IV-4

Figure IV-3 Thermal Denaturation of MS2-RNA in DMSO Solutions

MS2-RNA was diluted volumetrically into DMSO solutions of varying concentration and the absorbancy read as a function of the temperature. The percent concentration of DMSO (v/v) is given in the figure.

Figure IV-4 Thermal Denaturation of su-11-RNA in DMSO Solutions.

su-11-RNA was diluted volumetrically into DMSO solutions of varying concentration and the absorbancy read as a function of the temperature. The percent concentration of DMSO (v/v) is given in the figure.

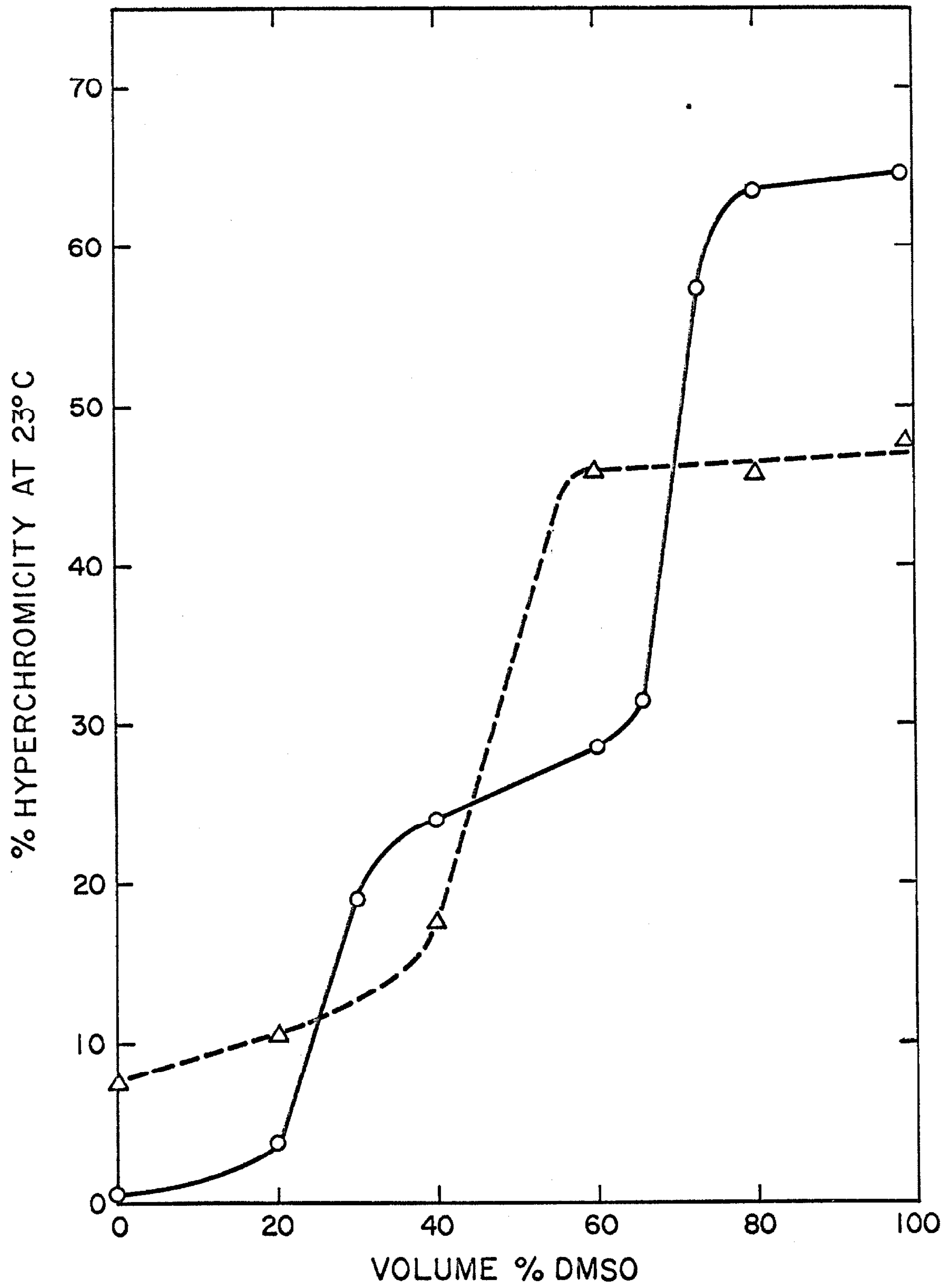

Fig. IV - 5

Figure IV-5 Denaturation in DMSO at Room Temperature

The percent hyperchromicity at 23°C is given as a function of the DMSO concentration.

O-O su-11-RNA

Δ-Δ MS2-RNA

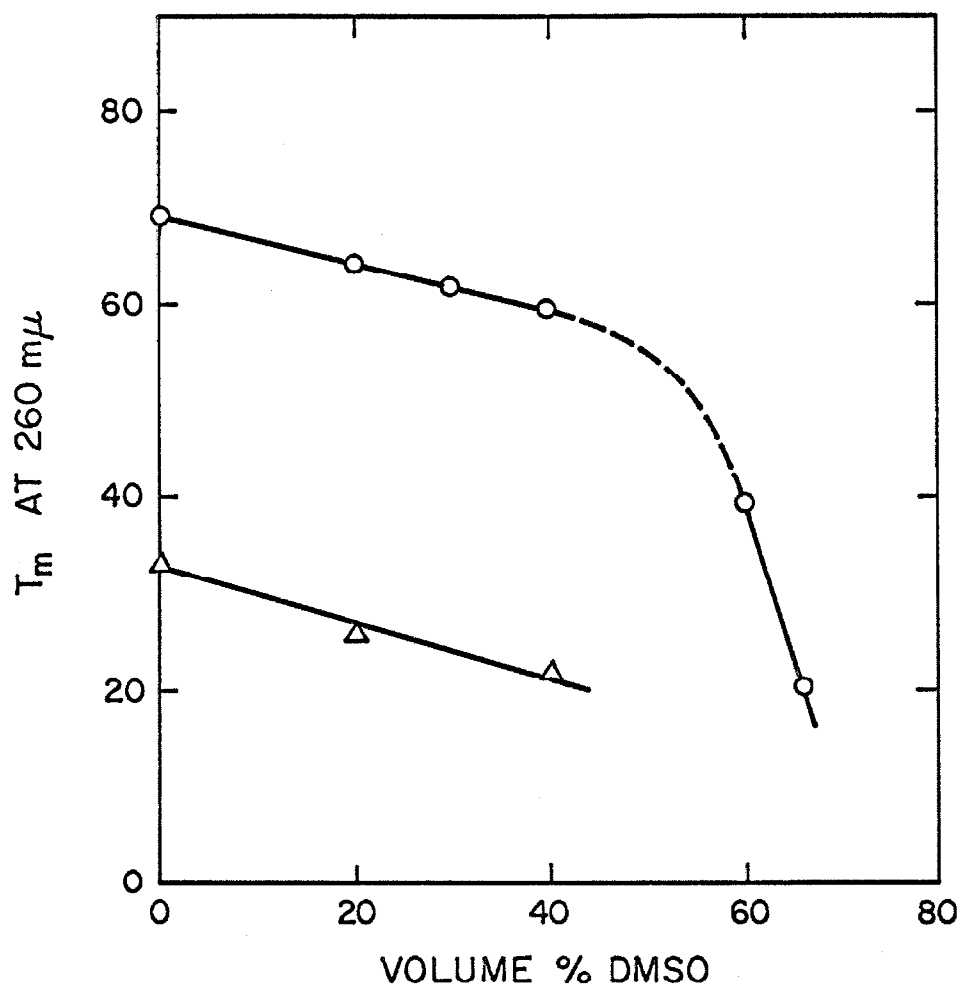

Fig. IV - 6

Figure IV-6 Effect of DMSO upon Thermal Denaturation

The T_m's measured from Figures IV-3 and IV-4 are given as a function of the DMSO concentration.

O-O su-11-RNA

Δ-Δ MS2-RNA

DMSO at this temperature; around 80% DMSO is required for complete denaturation of su-11-RNA.

There are two sharp transitions in DMSO at 23°C for su-11-RNA. The one having a midpoint at 70% DMSO can be identified as due to double-stranded RNA. The relationship of the transition having a midpoint at 26% DMSO (amounting to about 40% of the total hyperchromicity obtained) to the thermal transition pattern (Figure IV-2) is not entirely clear. From its magnitude, however, it would seem that it must comprise most of the material which gives a gradual thermal transition.

The hyperchromicities measured in DMSO are larger than those obtained in aqueous solution (Figures IV-3 and IV-4). The spectra of the individual nucleotides in 97% DMSO at 25°C were determined to see what effect this solvent has upon their absorption. The data for three of the nucleotides and of a calculated equimolar mixture are shown in Figure IV-7. The effect observed is a shift of the spectrum to longer wavelengths for three of the nucleotides; the spectrum of UMP is virtually identical in the two solvents. This leads to a 23% increase in absorption in DMSO at 280 mμ for the equimolar mixture, while the absorption at 260 mμ is almost unchanged.

Some of the data for su-11-RNA and MS2-RNA are collected in Table IV-3. Hyperchromicities for both RNA's

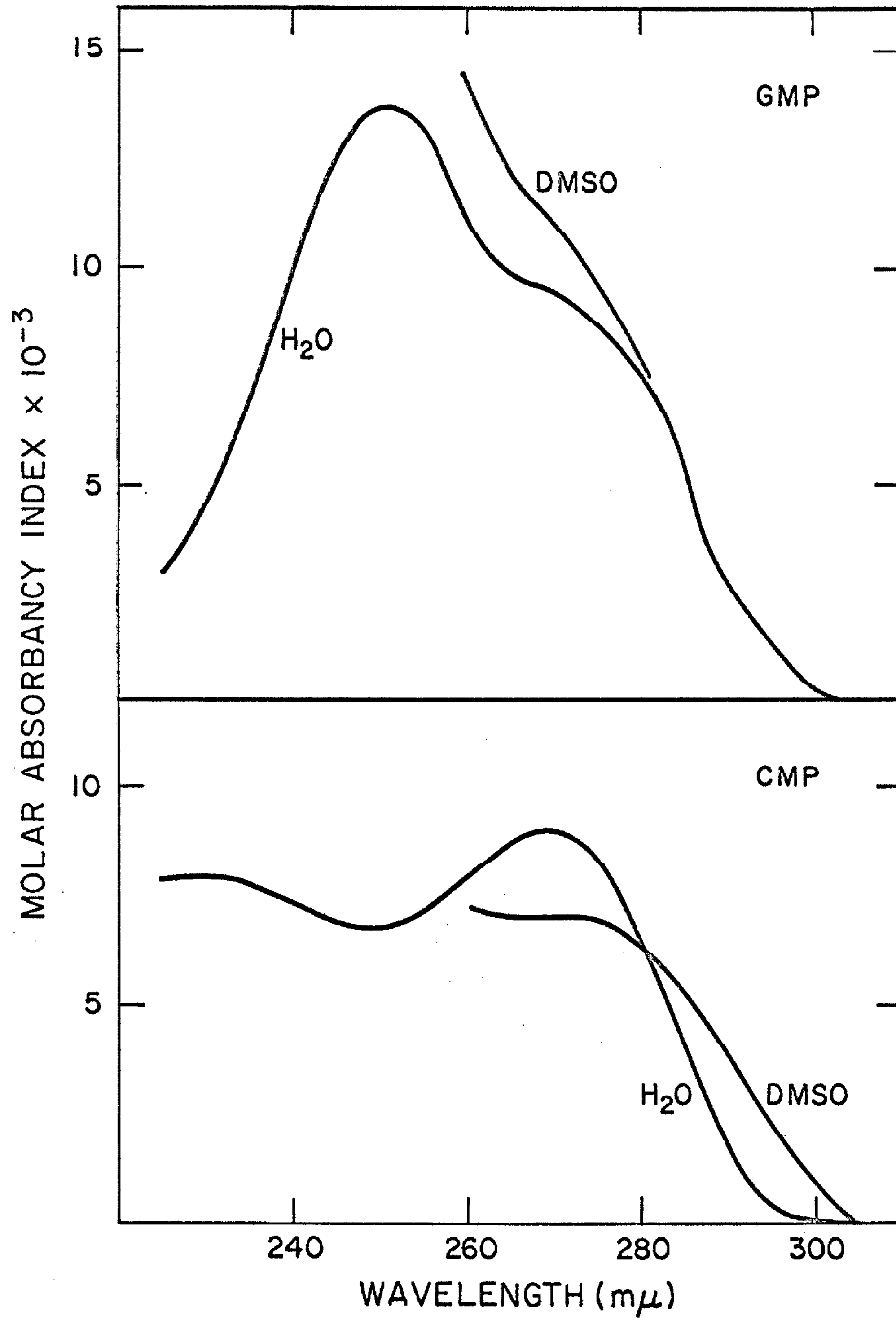

Fig. IV - 7

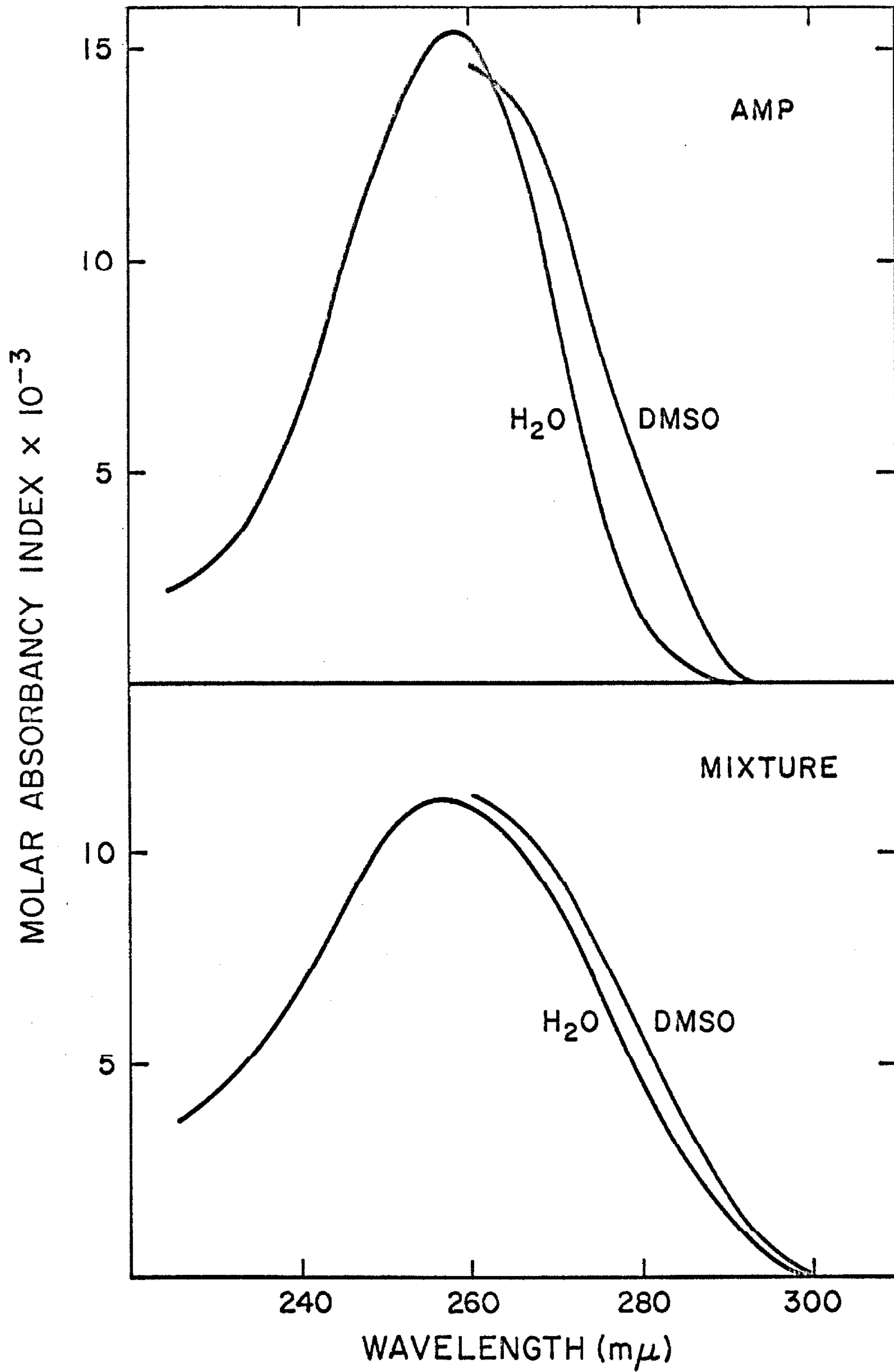

Fig. IV-7 (cont)

Figure IV-7 Effect of DMSO upon Absorption by the Nucleotides

The four nucleotides were diluted volumetrically into either SSC or into 97% DMSO (final concentration) containing 0.001 M EDTA pH 7.1, and the spectra recorded at 25°C with a Beckman DK2 Recording Spectrophotometer. The concentration of each nucleotide was determined from the absorption at 260 mμ in SSC and the results are expressed as molar extinction coefficients.

Table IV - 3

MS2-RNA and su-11-RNA in DMSO

Wavelength ($m\mu$)	su-11-RNA Hyper-chromicity	MS2-RNA Hyper-chromicity	MS2-RNA Extinc-tinn Coef-ficient	MS2-RNA Ratio to Nucleotides
280	93%	87%	6.2×10^3	1.10
270	80%	67%	9.8×10^3	1.04
260	65%	48%	11.3×10^3	0.99

All data are for 23°C. DMSO is 97-99% containing 0.001 M EDTA. The extinction coefficients of the nucleotides are those of Figure IV-7. For MS2-RNA the extinction coefficient was taken as 7.7×10^3 at 260 $m\mu$ in SSC.

in DMSO are given at three wavelengths. Taking the specific absorption of MS2-RNA as 22.2/mg/ml., the extinction coefficients of this RNA are listed in SSC and in DMSO; also given is the ratio of the RNA extinction coefficient to that of an equimolar mixture of the four nucleotides for both solvents. For DMSO this ratio varies from 1.1 at 280 mμ to 1.0 at 260 mμ.

We have also studied the absorption properties of rG:rC in DMSO. Figure IV-8 gives the spectrum of this material in SSC and in 95% DMSO. In Table IV-4 are given the extinction coefficients at three wavelengths of rG:rC in both aqueous and DMSO solution. Also given for both solvents are the ratios of the extinction coefficients of rG:rC to those of an equimolar mixture of the two nucleotides. In DMSO this ratio varies from 0.97 at 280 mμ to 0.88 at 260 mμ. The variation may not be significant. The rG:rC may contain an excess of cytidylic acid (9) which absorbs less than guanylic acid at the shorter wavelengths involved. In addition limited quantities of material were available.

In DMSO the observed ratios of polynucleotide extinction coefficients to those of the nucleotides are 12% less for rG:rC than the MS2-RNA. Whatever the reason for this, rG:rC appears largely or fully denatured, as evidenced by the large spectral shift and the hyper-

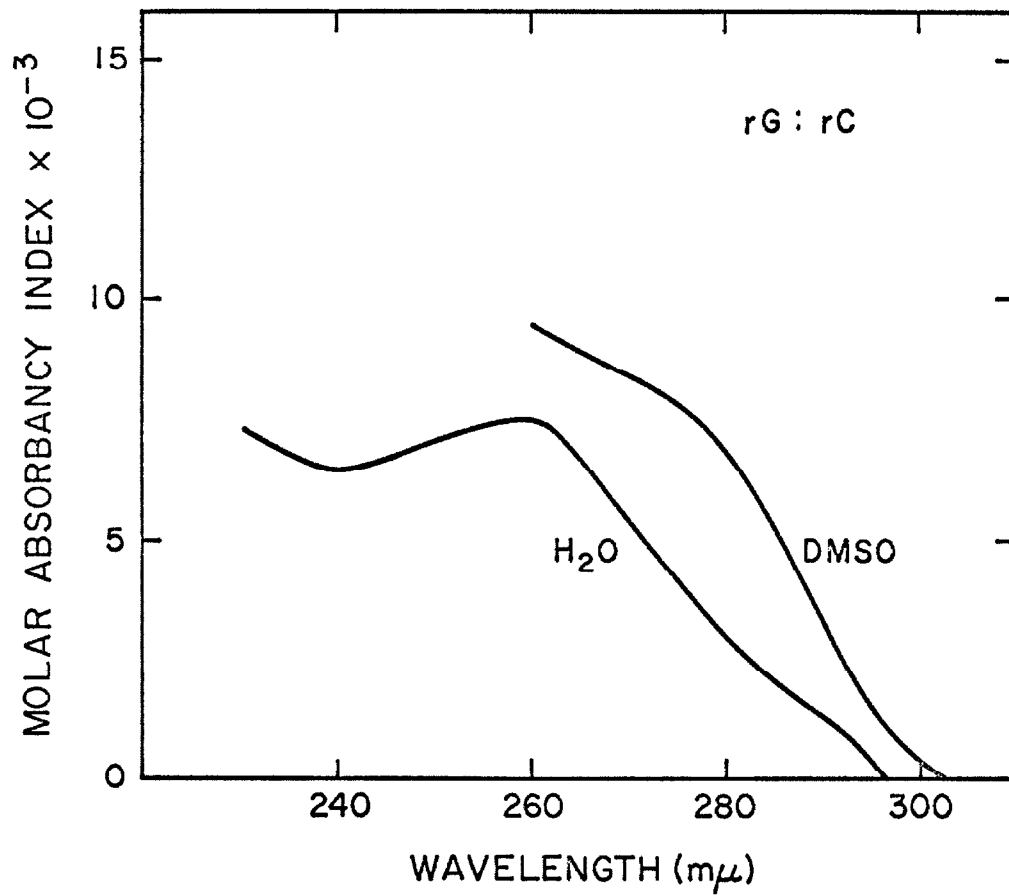

Fig. IV - 8

Figure IV-8 Absorption Spectra of rG:rC in Aqueous and DMSO Solution

The absorption spectrum of rG:rC was taken in SSC and in 94% DMSO containing 0.001 M EDTA pH 7 and 0.002 M LiCl. The concentration of material was determined from the absorption at 260 mμ in SSC and the results are expressed as molar extinction coefficients. The temperature was 25°C.

Table IV - 4

Extinction Coefficients of rG:rC in Aqueous and DMSO Solution

Wavelength	SSC		DMSO	
(mμ)	Extinction Coefficient	Ratio To Nucleotides	Extinction Coefficient	Ratio to Nucleotides
280	3.0×10^3	0.44	6.9×10^3	0.97
270	5.4×10^3	0.59	8.4×10^3	0.93
260	7.5×10^3	0.79	9.5×10^3	0.88

DMSO is 94% containing 0.001 M EDTA pH 7 and 0.002 M LiCl in the case of rG:rC and 97% containing 0.001 M EDTA pH 7 in the case of the nucleotides. The ratio given is that of the extinction coefficient of rG:rC to that of an equimolar mixture of CMP and GMP in the same solvent. The extinction coefficients are those of Figures IV-7 and IV-8.

chromicity produced. In particular the hyperchromicity is 130% at 280 mμ (the absorption of the nucleotides at this wavelength is almost the same in DMSO as in aqueous solution).

Denaturation in Formamide

The hyperchromicities observed with MS2-RNA and su-11-RNA in formamide (containing 0.01 M tris pH 7) are given in Table IV-5. For MS2-RNA this hyperchromicity is greater than shown in aqueous solution at 100°C (Table IV-1) but less than that shown in DMSO (Table IV-3). MS2-RNA thus appears to be largely or completely denatured in this solvent. The hyperchromicity of su-11-RNA is less than that of MS2-RNA in formamide and less than that shown by su-11-RNA at 100°C in aqueous solution. It, then, can be only partially denatured.

Attempts to study the hyperchromicity in formamide as a function of the temperature proved unsatisfactory. Upon heating either su-11-RNA or MS2-RNA in formamide, a fairly large, irreversible hyperchromicity increase was observed. A second heating cycle produced a further increase in hyperchromicity. Whatever is responsible for this effect does not lead to inactivation of MS2-RNA infectivity. The inactivation rate at 60°C (by which temperature the hyperchromicity increase has started) in 90% formamide containing 0.01 M tris pH 7 is comparable to

Table IV - 5

Hyperchromicity in Formamide

Temperature (^{o}C)	Hyperchromicity at 280 mμ	
	su-11-RNA	MS2-RNA
8	33%	51%
25	34%	57%

MS2-RNA was in 96% FA containing 0.01 M tris pH 7; su-11-RNA was in 93% FA containing 0.01 M tris pH 7.

that in 0.01 M phosphate pH 7.

DISCUSSION

The results shown in Figure IV-5 for su-11-RNA in DMSO are very similar to those found by Kelly (personal communication), who used the disappearance of the 280 mμ ORD peak as a criterion of denaturation. The disappearance of this peak is further evidence that RNA is denatured in DMSO. In this case the ORD peak is diminished by 20% in the DMSO concentration range of 20 to 40%, whereas the remaining 80% of the peak disappears at 60 to 80% DMSO.

Kelly (personal communication) has also found that the double-stranded form of MS2-RNA isolated after infection becomes infective to bacterial protoplasts after passage through high concentrations of DMSO, again indicating denaturation in this solvent.

Finally, rG:rC is the most resistant of nucleic acids and their analogues to denaturation. It also appears fully denatured in DMSO. These data, then, are all in agreement that RNA's, double- or single-stranded, are completely denatured in this solvent.

The concentration of DMSO required for denaturation appears to be related to the T_m of the RNA in aqueous solution. In addition to the data above for MS2-RNA and

su-11-RNA (which cannot be too closely compared because one is single-stranded, the other double-stranded), we note that Kelly (personal communication) has found that *E. coli* ribosomal RNA is "half-denatured" by 28% DMSO at room temperature. This RNA has a T_m in 0.1 M phosphate (comparable to SSC in ionic strength) of 54°C. (16).

The results in formamide are less complete. Single-stranded MS2-RNA appears to be denatured in high concentrations of this solvent, whereas su-11-RNA is not denatured. It is known that double-stranded DNA is denatured in formamide (7, 17). We have also shown that the replicative form of ØX174-DNA is denatured in formamide (18).

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PART V

SEDIMENTATION PROPERTIES OF MS2-RNA

INTRODUCTION

For some time we have observed the presence of two sedimenting components in MS2-RNA preparations when sedimentation was conducted at room temperature in 0.01 M or 0.02 M salt. This led us to search for a ring form for MS2-RNA analogous to that of the single-stranded DNA of bacteriophage ØX174 (1). For this purpose we studied denaturation conditions for the RNA and the kinetics of biological inactivation by RNase and alkali.

The actual or potential formation of ring structures appears to be a general phenomenon in the case of DNA viruses. Thus ØX-DNA is a covalently bonded ring (1, 2); upon infection it is converted into a double-stranded form which is also a covalent ring (3). The double-stranded DNA extracted from the animal viruses polyoma (4) and shope papilloma (5) are rings. DNA isolated from phage lambda is capable of forming hydrogen-bonded rings (6,7); upon infection it is converted into a covalently bonded ring (8). Finally, the DNA of T2 and T4 contain terminal redundancies and are potentially capable of forming rings (9). Although actual or potential ring molecules, if present, might serve a different function in the case of RNA viruses, it was of interest to determine if this principle extends to RNA.

Two sets of observations concerning this problem have appeared. Sugiyama (10) has found that MS2-RNA possesses a free 3'-terminal adenosine, and evidence has been presented that the RNA of turnip yellow mosaic virus is a ring or a partial ring (11, 12).

MATERIALS AND METHODS

Nuclease Inactivation

RNA for inactivation has been used at concentrations of 5 to 200 A_{260}/ml. The RNA sample (10 to 100 λ) is mixed with an equal volume of pancreatic RNase solution. The concentration of RNase used is calculated from the turnover numbers in Part III to give the desired number of hits in the solvent and time period used. Incubations were at 37°C, usually for one hour in the presence of 0.01% BSA, although some inactivations were for 30 minutes in the absence of BSA.

After inactivation the sample was chilled and an aliquot removed for assay of surviving infectivity by the methods of Part II. The RNA sample was then diluted into the desired solvent for analytical or preparative centrifugation.

ØX-DNA was a gift of Dr. R. L. Sinsheimer. For DNase inactivation 25 λ of DNA at 98 A_{260}/ml. in 0.1 M NaCl containing 0.05 M tris pH 8 was mixed with 25 λ of

pancreatic DNase solution. The DNase solution was 10 to 60 ng/ml in 0.002 M $MgSO_4$ containing 0.02% BSA. After inactivation for one hour at 37°C the sample was chilled, an aliquot removed for infectivity assay (performed by the method of Guthrie and Sinsheimer, ref. 13), and the DNA then diluted into the desired solvent for analytical centrifugation.

Alkali Inactivation

20 λ of MS2-RNA at 208 A_{260}/ml. in 0.1 M phosphate pH 7 was mixed with 30 λ of 0.14 M NaOH to give a pH of 12.27 (as determined from a calibration curve). After reaction for one to 15 minutes at 0°C, the sample was neutralized by the addition of 50 λ of 0.14 M NaH_2PO_4. After removal of an aliquot for assay of surviving infectivity the RNA was diluted into the desired solvent for analytical centrifugation.

Analytical Centrifugation

All runs were performed in a Spinco Model E Ultracentrifuge with monochromatic ultraviolet irradiation. With aqueous solutions a wavelength of 265 mμ was chosen, for DMSO solution the wavelength was 275 mμ. In earlier runs a single-sector centerpiece was used and photographs taken; the films were traced with a Joyce-Loebl Mark III Microdensitometer. Later runs used a double-sector centerpiece and direct traces were obtained with the

Spinco Photoelectric Scanning System.

In some cases two boundary runs were performed simultaneously in the two sectors of a double-sector centerpiece. The Photoelectric Scanning System traces the absorption in one sector as compared with the other sector. In this way small differences in sedimentation coefficient are readily visible.

Band centrifugation (14) was performed using a single- or double-sector Type III band-forming centerpiece (15), Kel F or charcoal-filled epon respectively. Unless otherwise stated DMSO is 99% containing 0.001 M EDTA pH 7.1. The sample to be layered over the DMSO was at 80 to 150 γ/ml. in 50 to 90% dimethylformamide containing 5×10^{-4} M EDTA and variable amounts of water and DMSO. Aqueous solvents for band centrifugation contained 90% D_2O, and the sample to be overlayered was in H_2O solution at a concentration of 60 to 150 γ/ml. In any case, 20 λ of sample was overlayered in single-sector centerpieces and 12 λ in double-sector centerpieces.

All analytical centrifugation in this section was conducted at 25°C. The uncorrected sedimentation coefficients in DMSO are given. In the case of aqueous solutions the sedimentation coefficients are corrected to water at 20° with the exception that no correction has been made for viscosity and density changes due to formaldehyde.

For boundary centrifugation sedimentation coefficients were calculated for the motion of the mid-point of the boundary. In the case of band centrifugation each peak was divided into two equal areas by a vertical line and this center of mass used to calculate the sedimentation coefficients.

Sucrose Gradients in DMSO

Preparative centrifugation through DMSO was performed with a stabilizing gradient of sucrose and, in one case, deuterated DMSO. Sucrose was dissolved in 99% DMSO containing 0.001 M EDTA pH 7.1; the sucrose concentration is expressed as w/v. The sucrose gradients used in DMSO have been 0 to 10%, 0 to 15%, 0 to 20%, and 5 to 20%. Because of the steep viscosity dependence on sucrose concentration in DMSO, the first is theoretically preferred; however, the stability is less and we have been unable to use a 0 to 10% gradient (alone) with an SW-65 rotor. The best result has been obtained with a 0 to 10% sucrose gradient which also contains a 0 to 75% gradient of deuterated DMSO.

Over each five ml. gradient was layered 0.05 to 0.1 ml. of 50% dimethylformamide, 25% DMSO, 25% water containing the RNA sample. Centrifugation was at 25°C.

Formamide Gradients

Formamide gradients were 80 to 94% or 80 to 98%

formamide containing 0.01 M tris pH 7.4. T' sample was overlayered in 0.1 ml. of aqueous solution. Centrifugation was at 5° or at $25^{o}C$.

Aqueous Sucrose Gradients

Aqueous sucrose gradients were 5 to 20% (w/w) containing various buffers. Centrifugation was usually at 5° or $25^{o}C$.

Formaldehyde Denaturation

The nucleic acid sample to be formaldehyde denatured was at 60 to 100 γ/ml. in HMP containing 1.8% formaldehyde. It was heated to $80^{o}C$ for 6 minutes. Sedimentation followed through 90% D_2O containing 1.8% formaldehyde and either HMP or 0.02 M phosphate pH 7.

Preparation of C^{14}-Labelled MS2-RNA

E. coli C3000 was grown at $37^{o}C$ to a cell concentration of 2×10^{8}/ml. in 200 ml. of TPA medium (16) containing 5 γ/ml. of uracil. The cells were spun out in the cold and resuspended in 200 ml. of prewarmed TPA. 0.2 mc of uracil-2-C^{14} at a specific activity of 28 mc/mM was added (final uracil concentration of 4 γ/ml.) and the culture infected with MS2 at a multiplicity of infection of about 10. Lysis started 1.5 hours later, and the culture was removed 2.5 hours after infection. The lysate titer was 8×10^{11}/ml.

Purification of the virus proceeded as described in

Part I, suitably scaled down for the smaller volume. 1.1 mg of virus were isolated of which 21% were active plaque-formers. The specific activity of the pyrimidines in the preparation was 17 mc/mM, or 60% of the specific activity of the uracil used.

RNA was isolated from the virus as described in Part I, and stored at -70°C. The specific activity of the RNA was 1.0×10^{-7} dpm/RNA molecule.

Preparation of TMV-RNA

TMV was prepared from infected tobacco leaves by the method of Simmons, as described in Friesen and Sinsheimer (17). RNA was isolated from the virus by the method of Fraenkel-Conrat *et al*. (18).

Ribosomal RNA's

Lysates of *E. coli* C3000 were kindly provided by Dr. G. N. Godson, prepared by lysozyme-EDTA treatment followed by lysis with a neutral detergent (19). After spinning out the cell debris, 6.7 ml. of lysate per tube were underlayered with 4 ml. of 30% sucrose containing 0.08 M NaCl, 0.01 M tris (pH 7.6), and 0.01 M $MgSO_4$, and the ribosomes pelleted in a 40-rotor.

The ribosomes were resuspended in 0.08 M NaCl containing 0.01 M tris (pH 7.6) and 0.01 M $MgSO_4$. Sodium dodecyl sulfate was added to 2%, and the mixture extracted three times with phenol. The RNA was precipitated with

ethanol, resuspended in 0.1 M tris (pH 7.0) containing 0.01 M EDTA, then twice more precipitated. The final precipitate was taken up in 0.001 M EDTA pH 7.1 and stored at -20°C. The RNA preparations contained a weight ratio of 23S to 16S RNA of 2.0-2.1 to 1 when analyzed by analytical centrifugation.

Rabbit reticulocyte ribosomal RNA was a gift of Dr. E. R. Glowacki; the preparation and properties of this RNA are described by her (20).

Abbreviations Used

DMSO = dimethylsulfoxide

HMP = 0.01 M sodium phosphate, pH 7.0

TMV = tobacco mosaic virus

RESULTS

Molecular Weight Dependence of Sedimentation

We have studied the sedimentation properties of six RNA's in the solvents used in this section. These solvents are HMP, HMP containing 1.8% formaldehyde (in which case the RNA has been previously reacted with formaldehyde), and 99% DMSO containing 0.001 M EDTA (pH 7.1). The sedimentation coefficients in DMSO show a simple dependence on molecular weight; in the aqueous solvents they do not.

The RNA samples used were MS2-RNA, TMV-RNA, *E. coli*

ribosomal RNA, and rabbit reticulocyte ribosomal RNA. Some of the data for the last named RNA are taken from Glowacki (20).

The molecular weight of TMV-RNA is 2.0 to 2.1 x 10^6 (21). The molecular weights for the E. coli ribosomal RNA's have been reported as 0.55 and 1.07 x 10^6 for the potassium salt (22) and as 0.56 and 1.1 x 10^6 (23) (asumed to be for the sodium salt). The molecular weights of the rabbit reticulocyte ribosomal RNA's are less well established. Hall and Doty (24) found values of 0.6 and 1.3 x 10^6 for the ribosomal RNA's of calf liver, Petermann and Pavlovec (25) obtained molecular weights of 0.55 and 1.7 x 10^6 for the rat liver ribosomal RNA's, and Gierer (26) reported the molecular weights of the latter RNA's to be 0.6 and 1.8 x 10^6.

The results in DMSO are shown in Figure V-1. "28 S" rabbit reticulocyte RNA is entered twice, as bars at the two molecular weights found for mammalian ribosomal RNA's. The other RNA's are represented as rectangles which include the range of molecular weights found for that RNA and the range of sedimentation coefficients obtained or the estimated error in this quantity.

The line shown in Figure V-1 follows the equation:

$$S_{25,DMSO} = 0.052\ M^{0.31}$$

and provides a good fit to the data. Use of this equation

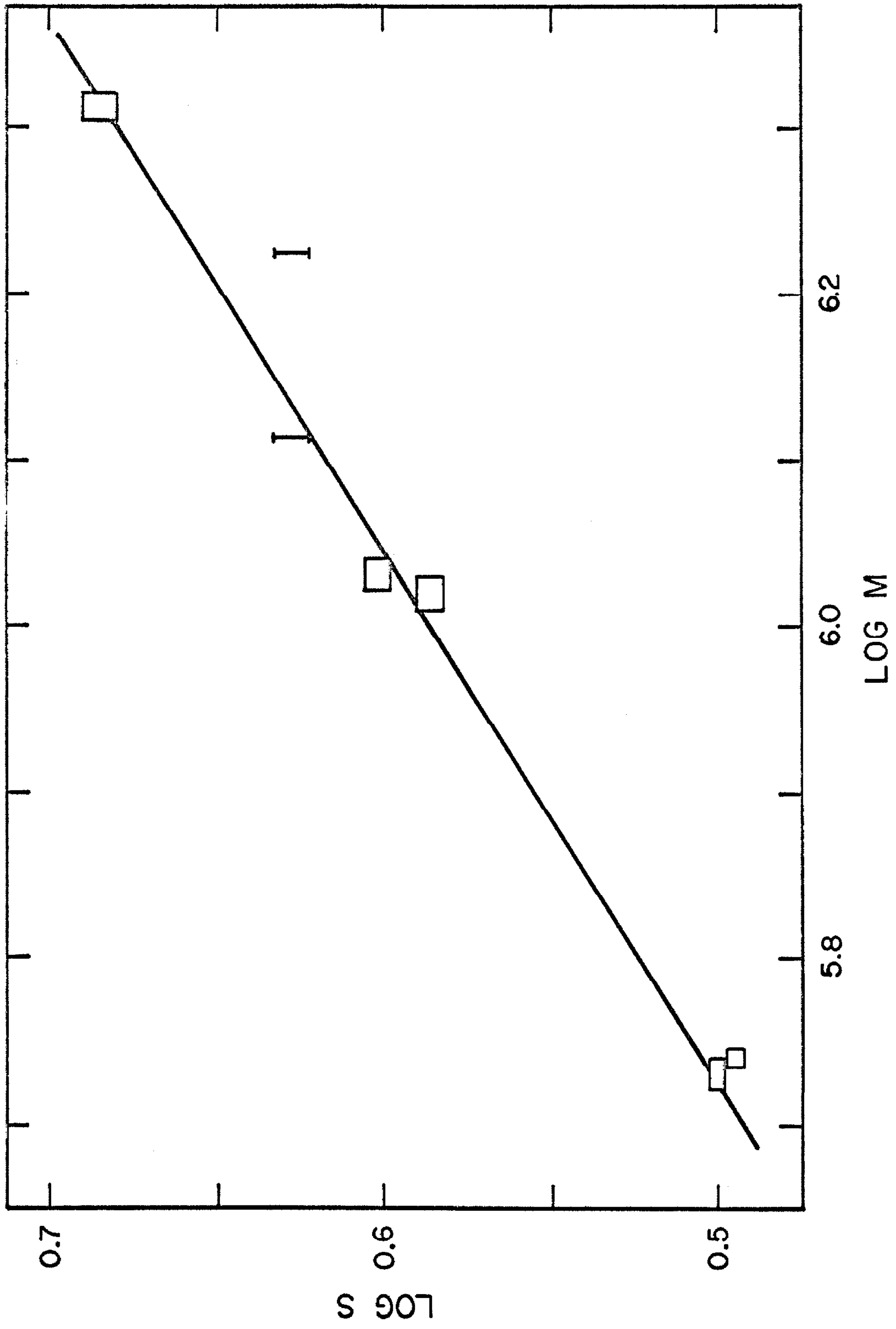


Fig V-1

Figure V-1 The Dependence of the Sedimentation Coefficient upon RNA Molecular Weight

Sedimentation coefficients in 99% DMSO containing 0.001 M EDTA pH 7.1 were determined by band centrifugation, and the uncorrected values are given as a function of the RNA molecular weight. Note that both scales are logarithmic. Some of the data for rabbit reticulocyte ribosomal RNA are taken from Glowacki (20).

gives molecular weights within 10% of the values reported for the various RNA's (leaving aside the larger mammalian ribosomal RNA). Therefore, sedimentation through DMSO appears to provide a reliable estimate of RNA molecular weight within the molecular weight range studied here.

If this equation is to be applied, it is important that the DMSO solution be fresh, for DMSO is very hygroscopic and the uncorrected sedimentation coefficient of MS2-RNA in 90% DMSO is 61% of that in 99% DMSO. Use of an unsealed sample of DMSO leads, then, to a progressive decrease in the observed sedimentation coefficients.

For the aqueous solvents used, no simple relation between sedimentation coefficient and molecular weight exists. The sedimentation coefficients are given in Table V-1. Note in particular that TMV-RNA sediments more slowly that reticulocyte "28 S" RNA. In the case of the ribosomal RNA's the sedimentation coefficient depends upon a higher power of the molecular weight than in DMSO solution.

Sedimentation of MS2-RNA under Denaturing Conditions

When MS2-RNA is sedimented through DMSO or through formaldehyde solution a single leading peak is observed followed by diffuse trailing material. This is also true after partial degradation of the preparation with RNase or with alkali. Representative patterns are shown in

Table V - 1

Sedimentation Coefficients of RNA's in Aqueous Solution

RNA	$S_{20,w}$	
	HMP	HMP + 1.8% CH_2O
Reticulocyte "16 S"	14.4	8.65
E. coli "16 S"	12.9	7.43
E. coli "23 S"	18.3	9.15
MS2	18.5, 20.8	9.40
Reticulocyte "28 S"	27.1	13.2
TMV	24.1	11.5

Sedimentation coefficients were determined by the method of band centrifugation. In the case of sedimentation through formaldehyde solution the RNA samples had been previously reacted with this reagent.

The RNA samples, in order of increasing molecular weight, are the 16 S component of rabbit reticulocyte ribosomal RNA, the 16 S component of *E. coli* ribosomal RNA, the 23 S component of *E. coli* ribosomal RNA, MS2-RNA, the 28 S component of rabbit reticulocyte ribosomal RNA, TMV-RNA.

Figure V-2. Under these sedimentation conditions the ring and open forms of ØX-DNA are resolved, as illustrated in Figure V-3.

The relationship between biological hits and the number of hits calculated from the sedimentation pattern is shown in Figure V-4. The RNA was inactivated at pH 12.27 and sedimented through DMSO, or inactivated with RNase and sedimented through HMP, formaldehyde solution, or DMSO.

When the RNA is degraded with RNase, the relationship between the biological hits produced and the increase in centrifugal hits is close to 1:1. This ratio of biological to centrifugal hits is the same for the two aqueous solvents used; reaction with formaldehyde reveals no additional chain scissions. This is not true for TMV-RNA, where we have observed differences with nuclease treated samples when sedimented through HMP and through formaldehyde solution.

The number of biological hits per centrifugal hit is higher when sedimentation is through DMSO (1.2) than when through aqueous solution (0.8). This difference may be due to an overestimation of the amount of material in the leading peak during DMSO sedimentation because the sedimentation coefficient changes slowly with molecular weight. We cannot explain the lower ratio in HMP as due

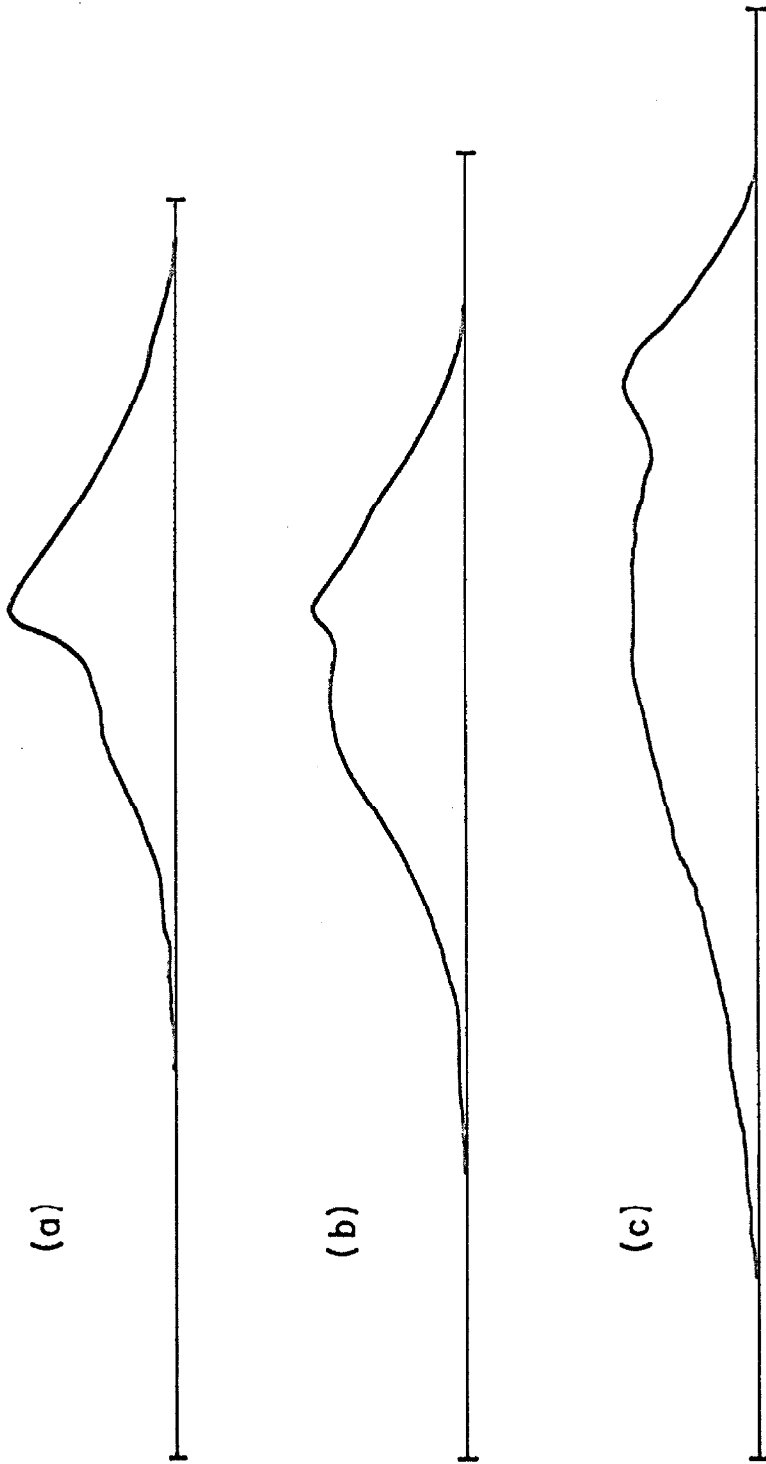


Fig. V - 2

Figure V-2 Sedimentation of MS2-RNA under Denaturing Conditions

(a) Pattern in DMSO after 112 minutes at 56,100 rpm.

(b) Pattern in DMSO after 196 minutes at 56,100 rpm. The RNA sample had received 0.7 biological hit at pH 12.27

(c) Pattern in HMP containing 1.8% formaldehyde after 80 minutes at 56,100 rpm. The sample had received 0.3 biological hit from RNase.

Sedimentation is from left to right.

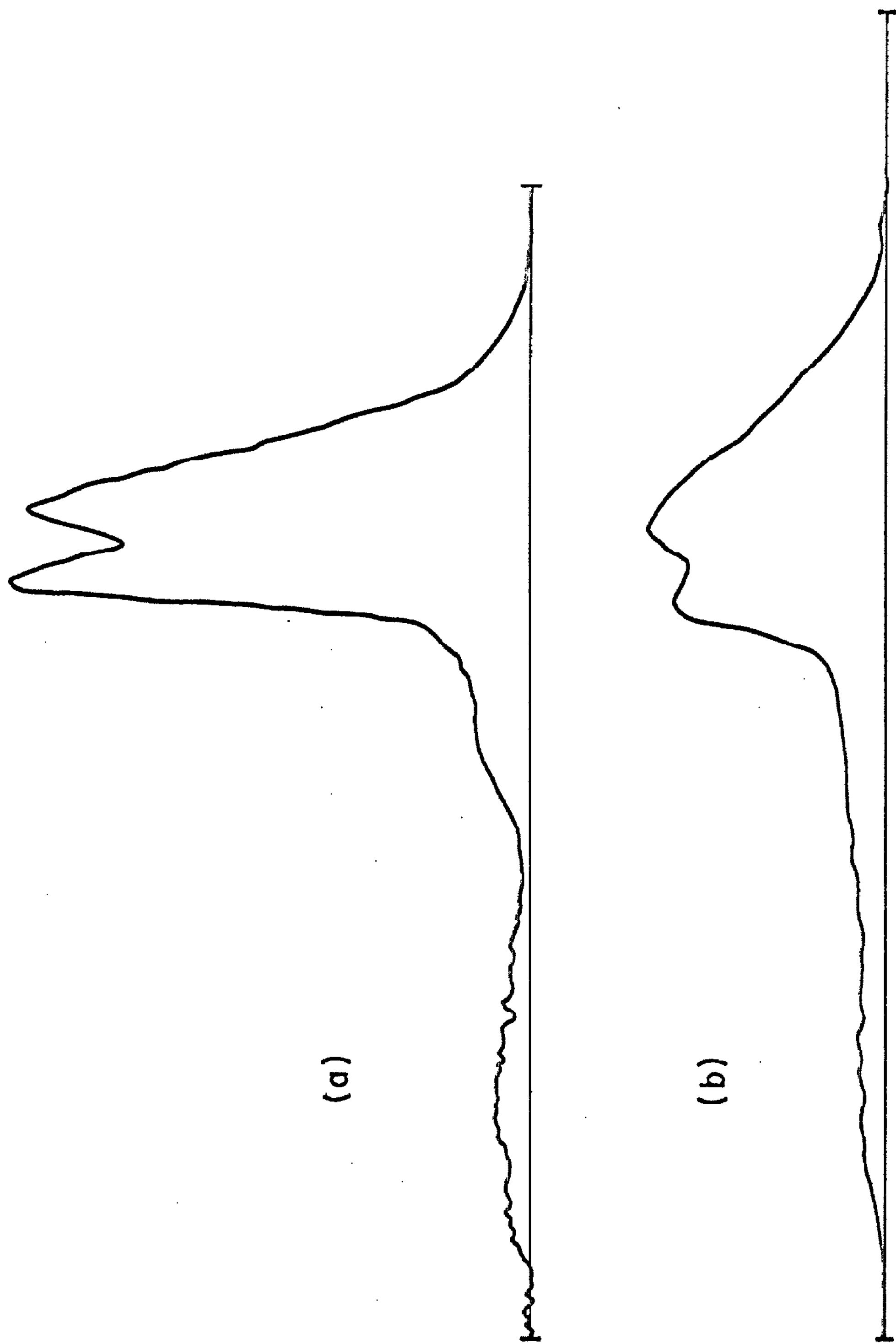


Fig. V - 3

Figure V-3 Sedimentation of ØX-DNA under Denaturing Conditions

(a) Pattern in DMSO after 128 minutes at 56,100 rpm. The sample had received 0.5 biological hit from DNase.

(b) Pattern in HMP containing 1.8% formaldehyde after 80 minutes at 50,740 rpm. The sample had received 0.5 biological hit from DNase.

Sedimentation is from left to right.

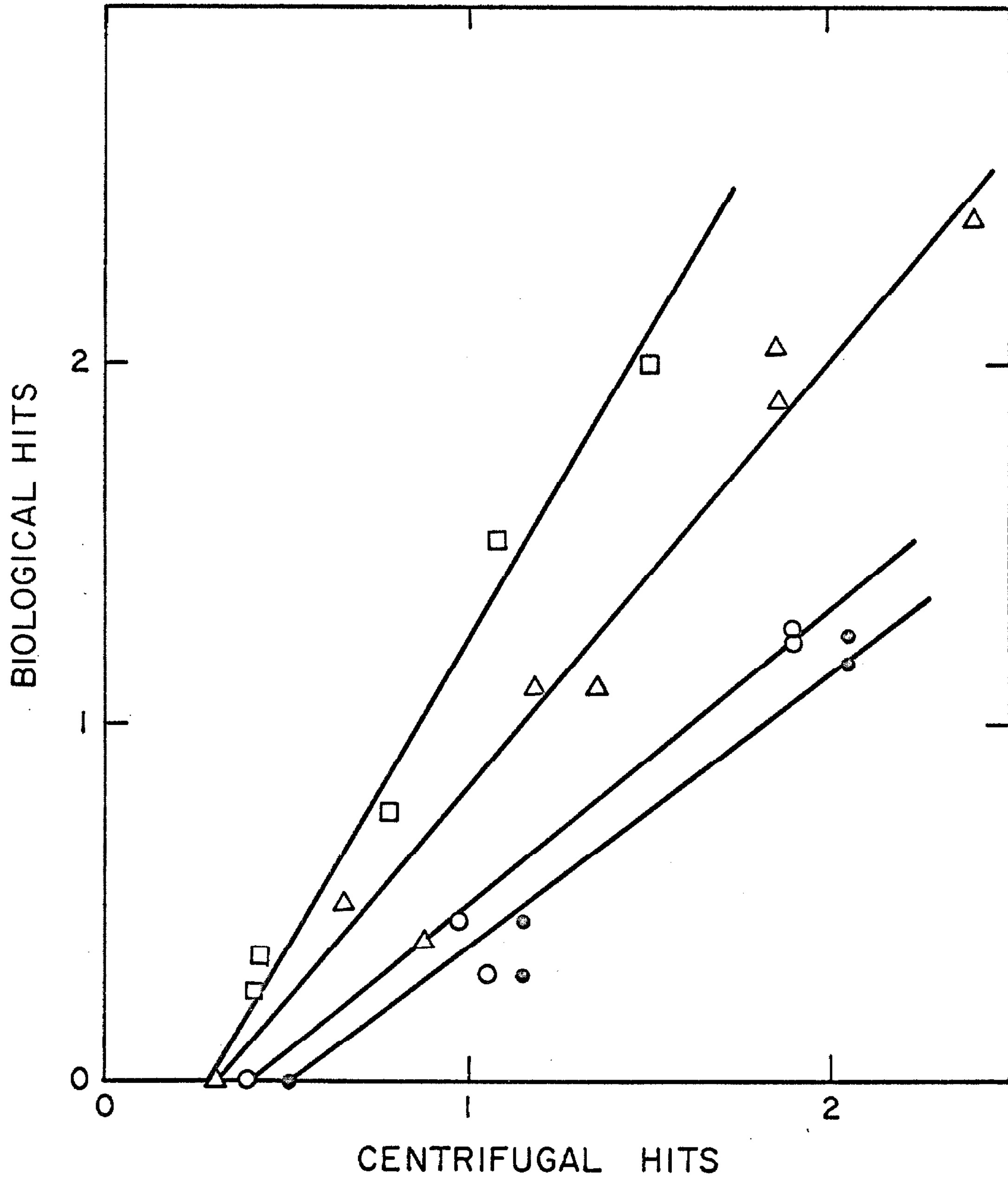


Fig. V - 4

Figure V-4 Biological Hits Compared with Centrifugal Hits

The fraction of RNA sedimenting in the leading peak was found by integration of areas; surviving infectivity was also determined for each sample. The number of hits, biological or centrifugal, was then determined from the zero order term of the Poisson distribution: $P_0=\exp(-m)$, where P_0 is the surviving infectivity or the fraction of RNA in the leading peak, respectively, and m is the number of hits.

●-● RNase inactivation, sedimentation through formaldehyde solution

O-O RNase inactivation, sedimentation through HMP

Δ-Δ RNase inactivation, sedimentation through DMSO

□-□ Inactivation at pH 12.27, sedimentation through DMSO

to continued action of RNase before or during the centrifugation. The extent of reaction with RNase in HMP at 25°C is insignificant compared to the initial reaction for 30 minutes at 37°C in 0.05 M phosphate.

For RNase inactivation, then, there are 1.0 ± 0.2 biological hits per centrifugal hit. From this and the absence of two forms we conclude that our MS2-RNA preparations consist of linear molecules.

After reaction with alkali, however, the number of biological hits exceeds the number of centrifugal hits; the ratio of the two (1.7) remains constant over a considerable range. There is a second reaction occurring under our conditions (accounting for about one-third of the biological hits) which inactivates the infectivity of MS2-RNA without chain cleavage.

ØX-DNA, inactivated to various extents with DNase, has been used as a control. The expected relationships among the surviving infectivity, the ring form, and the open form were observed.

Although our preparations of MS2-RNA consist of linear molecules, it is possible that the infective molecules are present in small proportion and do contain a covalent link joining the chain upon itself. To study this we have used preparative centrifugation through various solvents, comparing the infectivity with the bulk

RNA. These include sucrose gradients under a variety of conditions, formamide gradients, and sedimentation through DMSO. Under no conditions have we been able to demonstrate a separation of the infectivity from the peak of RNA as followed by radioactivity or optical density. (Under conditions of high RNA concentration, however, the trailing edge is not infective. This is probably due to concentration-dependent sedimentation, when degraded RNA sediments more rapidly for its size than the undegraded RNA because it is in a region of lower concentration).

The sucrose gradient shown in Figure V-7 shows multiple forms of MS2-RNA, all of which are infective. Thus sedimentation under these conditions is sufficient to resolve RNA molecules differing in configuration. Since the infectivity closely parallels the radioactivity peak, it is unlikely that the infective molecules differ in configuration or mass from the bulk of the RNA preparation.

MS2-RNA has been sedimented through DMSO stabilized with various density gradients. A representative gradient is shown in Figure V-5; the infectivity peak is coincident with the radioactivity peak.

Since the infectivity sediments with the RNA peak during preparative centrifugation under all conditions investigated, it is likely that the infective molecules do not differ from the bulk of the RNA (presumably all is

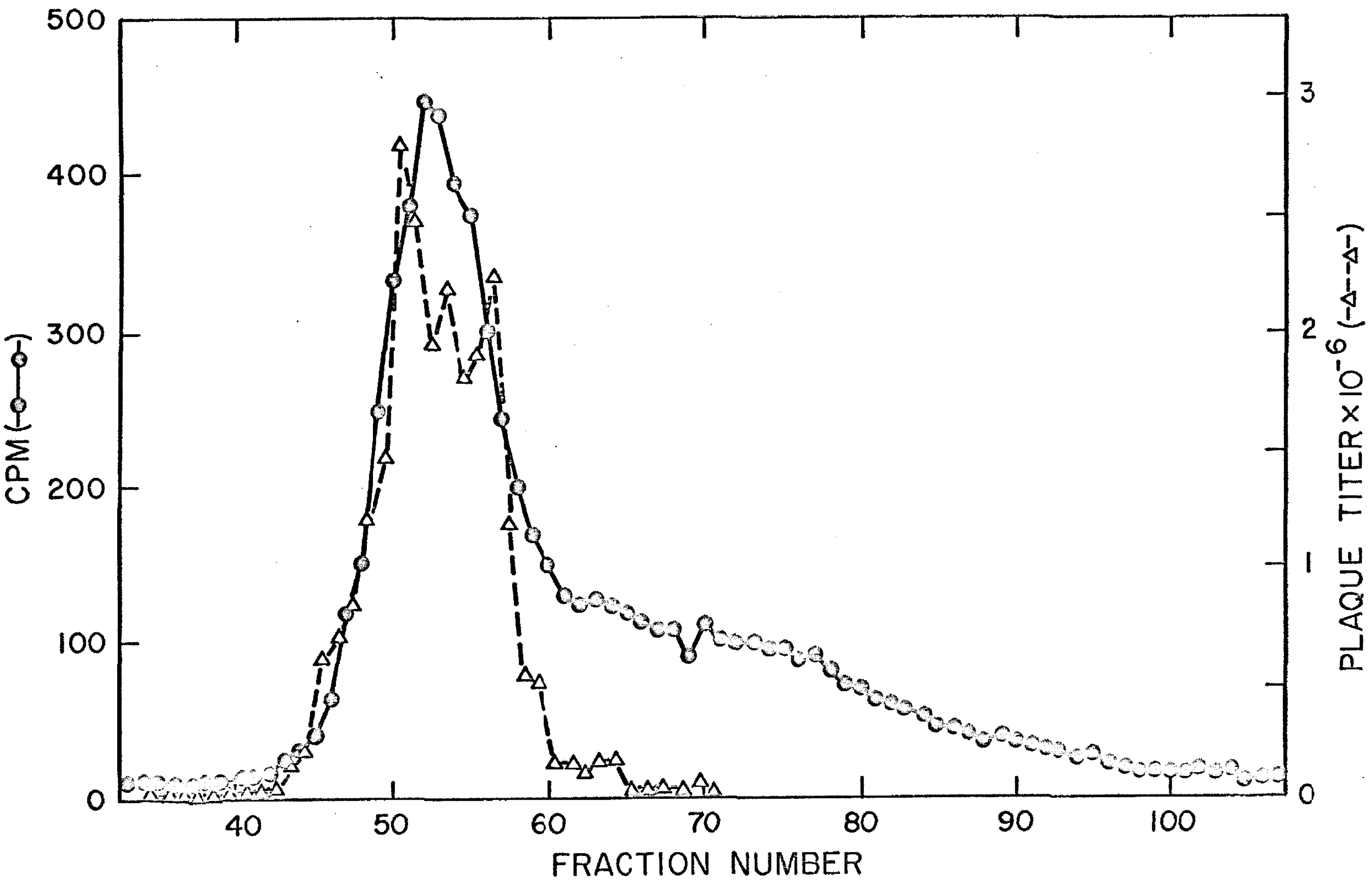


Fig. V -5

Figure V-5 Preparative Centrifugation of MS2-RNA through DMSO

C^{14}-MS2-RNA was sedimented through DMSO. The stabilizing density gradient was 0 to 10% sucrose and 0 to 75% deuterated DMSO. Sedimentation was for 10 hours at 25°C at 64,000 rpm in an SW-65 rotor. Four-drop fractions were collected, consisting of three drops directly onto planchets and one drop into 0.4 ml. of 0.01 M tris (pH 7) for infectivity assay. Only part of the gradient is shown; there was a total of 159 fractions. Sedimentation is from right to left.

●–● Radioactivity

Δ–Δ Infectivity

potentially infective), and are linear molecules.

Sedimentation in Non-denaturing Solvents

When MS2-RNA is sedimented through 0.01 or 0.02 M salt at 20° or 25°C, at least two components are seen. Results from both band and boundary centrifugation are shown in Figure V-6. (The ring and open form of ØX-DNA are resolved under these conditions.)

The results of three sucrose gradients in HMP at 25°C are shown in Figures V-7, V-8, and V-9. These three gradients contain varying ratios of two RNA preparations. The radioactive preparation appears to contain four components in all three gradients. In view of the reproducibility of this result we believe all four components are real. If so, each component is homogeneous for the total span in sedimentation coefficients among these components is 13% in the first two gradients. If the individual components were significantly heterogeneous in sedimentation coefficient we would not expect the resolution we observe.

The gradient in Figure V-7 contains the radioactive preparation alone, and shows all components to be infective. The gradient in Figure V-8 contains a 20-fold excess of a non-radioactive preparation; thus the infectivity is associated with this preparation. Based upon the fact that infectivity is found throughout the

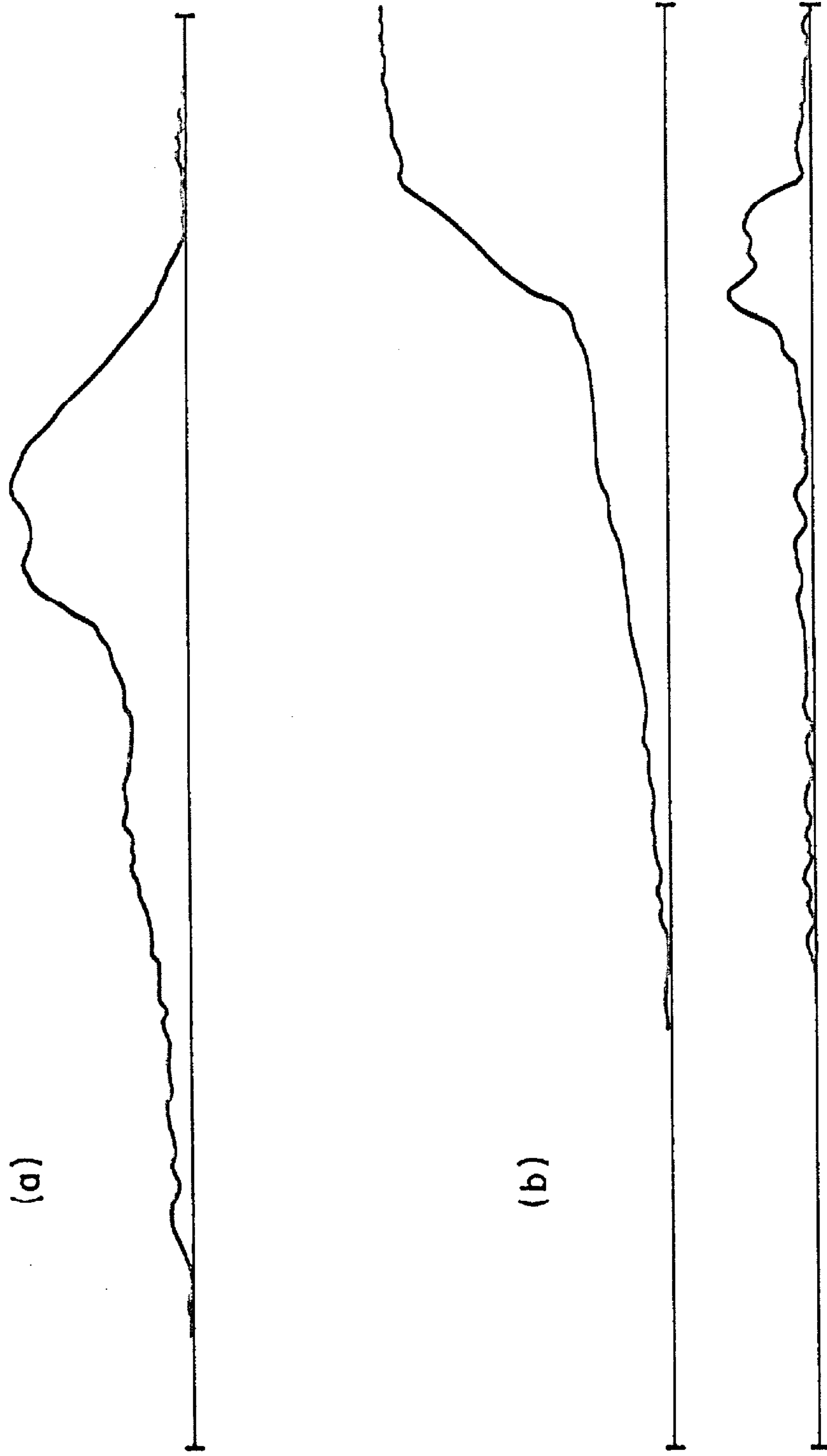


Fig. V-6

Figure V-6 Sedimentation of MS2-RNA through HMP at 25°C

(a) Band centrifugation.

(b) Boundary centrifugation. The derivative tracing is also shown below the optical density tracing.

Sedimentation is from left to right.

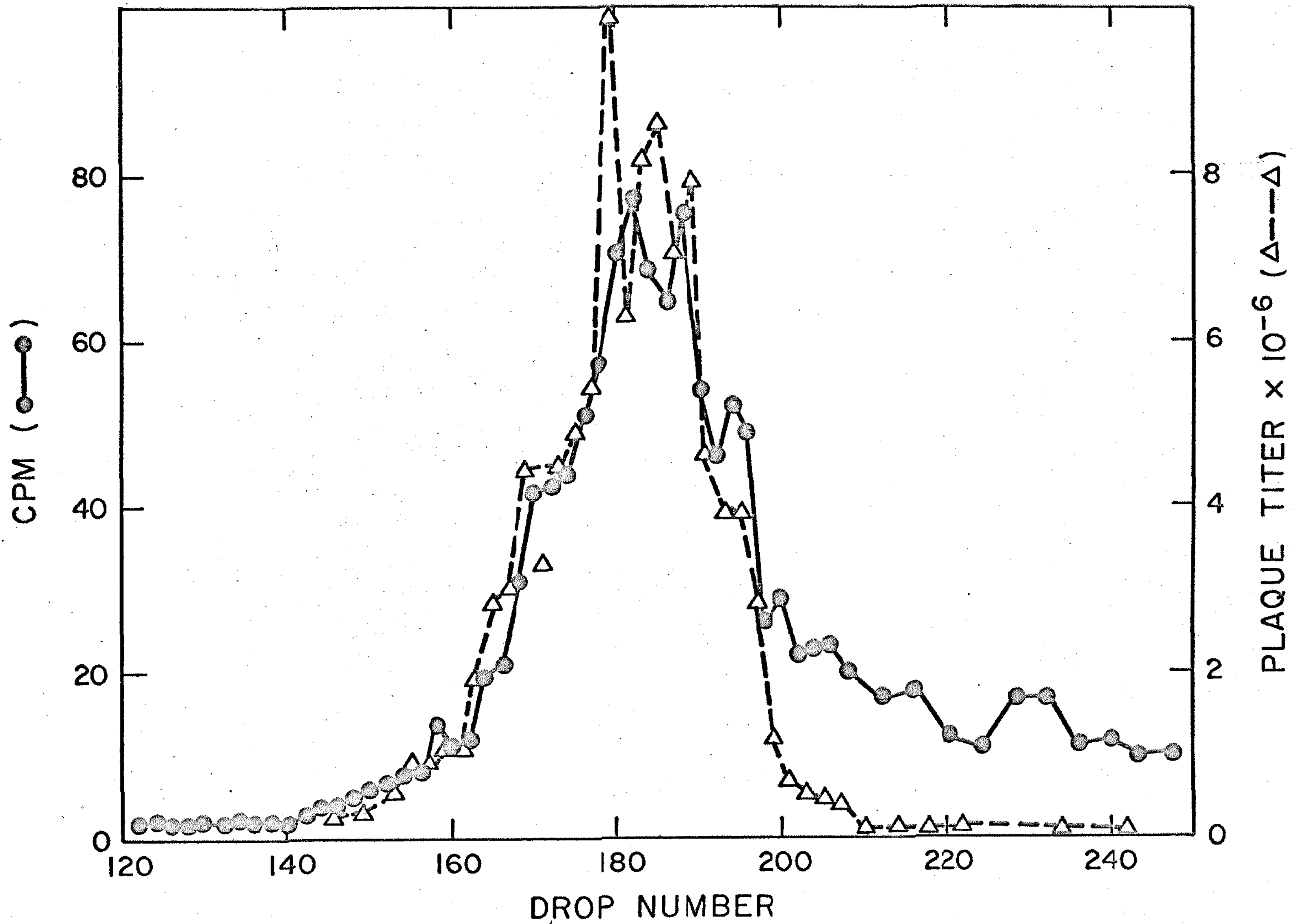


Fig. V-7

Figure V-7 Preparative Centrifugation of MS2-RNA through HMP at 25°C

0.5 γ of C^{14}-MS2-RNA was layered over a 5 ml. sucrose gradient containing HMP. Centrifugation was for 6 hours at 31,000 rpm at 25°C. A variable collection schedule was used, collecting drops alternately onto planchets and into 0.4 ml. of 0.05 M tris for infectivity assay. The entire gradient is not shown; there was a total of 360 drops. Sedimentation is from right to left.

●-● Radioactivity

Δ-Δ Infectivity

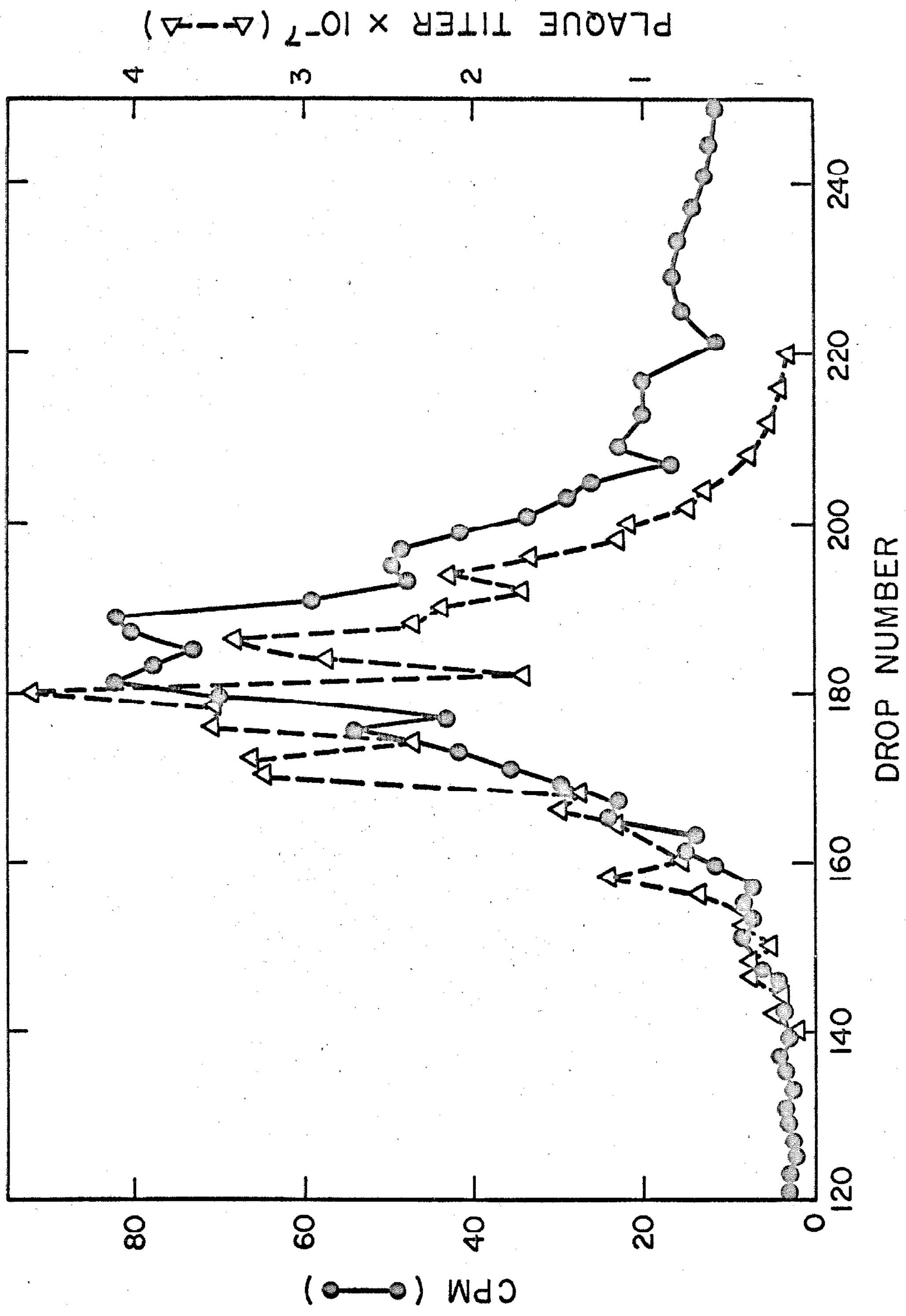


Fig. V-8

Figure V-8 Preparative Centrifugation of MS2-RNA through HMP at 25°C

0.5 γ of C^{14}-MS2-RNA and 10 γ of non-radioactive RNA were sedimented through a sucrose gradient containing HMP. Centrifugation was for 6 hours at 31,000 rpm. A variable drop collection schedule was used, collecting drops alternately onto planchets and into 3.0 ml. of 0.05 M tris (pH 7) for infectivity assay. The entire gradient is not shown; there was a total of 360 drops. Sedimentation is from right to left.

●-● Radioactivity

Δ-Δ Infectivity

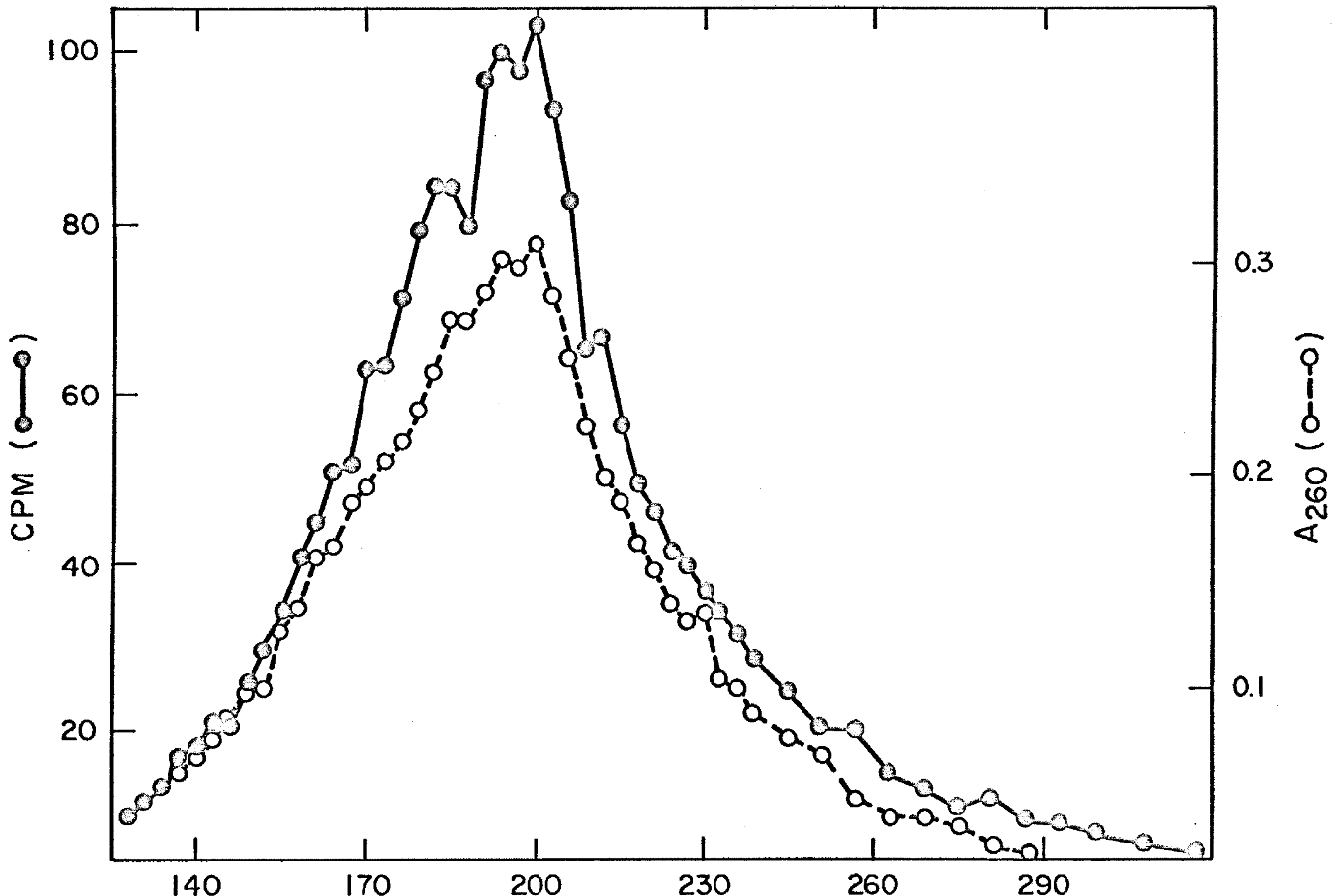


Fig. V-9

Figure V-9 Preparative Centrifugation of MS2-RNA Through HMP at 25°C

0.5 γ of C^{14}-MS2-RNA and 200 γ of a non-radioactive preparation were sedimented through a sucrose gradient containing HMP. Centrifugation was for 6 hours at 31,000 rpm. Three drop samples were collected into 0.6 ml. of 0.05 M tris pH 7. The optical density of the samples was measured, and after removal of an aliquot for assay of infectivity the entire sample was placed onto planchets. The infectivity data were more scattered than in the preceding two figures, and have been omitted to avoid crowding the figure. In general the infectivity paralleled the peak except that the leading edge appeared more infective than the trailing edge (which could be due to concentration-dependent sedimentation). The entire gradient is not shown; there was a total of 360 drops. Sedimentation is from right to left.

● - ● Radioactivity

O - O Optical density

peak region the non-radioactive preparation also contains multiple forms. The gradient in Figure V-9 contains a 400-fold excess of the non-radioactive preparation; the peak region is considerably broadened by concentration-dependent sedimentation.

The origin of these bands has been further investigated in a series of analytical runs in which the RNA had been heated under various conditions. The experimental data was difficult to interpret because of the scatter in observed sedimentation coefficients and the small differences involved.

The results of three such runs are given in Table V-2 to illustrate the effects obtained. The heat treated RNA was run in one sector of a double-sector cell, the untreated RNA in the other sector. Thus only boundaries which are present in one sample but absent, or present in reduced amounts, in the other are recorded.

In the first run given in Table V-2, components of the unheated samples having sedimentation coefficients of 19.1 S and 17.9 S have been converted to a 17.4 S component by heating to 7°C above the T_m of the RNA. The components which are affected account for 64% of the RNA sample, or most of the boundary region (30% of the preparation is found in trailing material). The sedimentation pattern for this experiment is given in Figure

Table V - 2

Effect of Heating on MS2-RNA Components

Treatment	$S_{20,w}$'s Heated Sample	Untreated Sample
70°C, 3 minutes	17.4 (50%)	19.1 (42%), 17.9 (22%)
60°C, 6 minutes	18.3 (20%)	20.1 (12%), 19.1 (14%)
50°C, 20 minutes	17.9 (29%)	17.1 (29%)

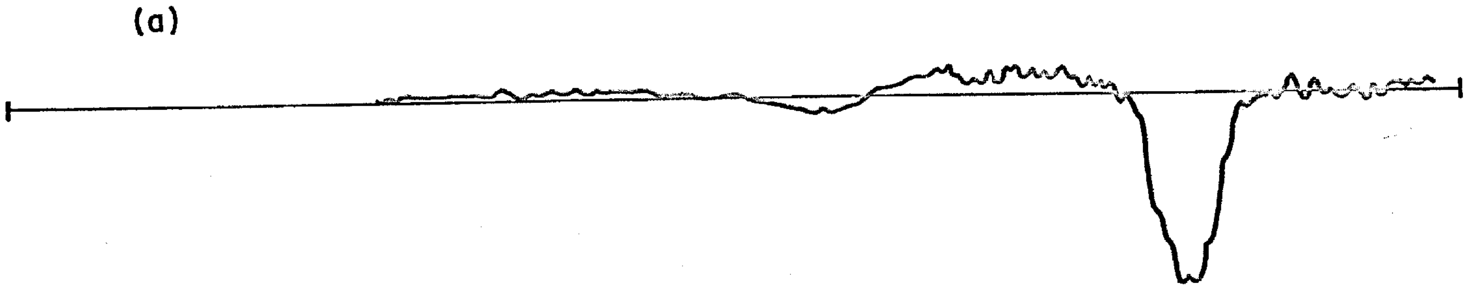
MS2-RNA at 7.7 A_{260}/ml. was heated in 0.11 M sodium phosphate; the time and temperature is shown in the table. The RNA was then diluted 11-fold to give a concentration of 0.7 A_{260}/ml. in HMP. This sample was placed into one of the sectors of a double-sector cell. Into the other sector was placed untreated RNA at the same concentration in HMP. Thus only boundaries of changed sedimentation coefficient are traced. In parentheses behind the sedimentation coefficient is given the percentage of RNA (relative to the total amount of RNA in each sector) sedimenting in that boundary.

V-10(b). In the second experiment, a similar conversion of two faster forms to a more slowly sedimenting form occurs after heating the RNA to just below its T_m; this conversion involves only 26% of the RNA sample, however. In the third experiment, where the RNA is heated to 13°C below its T_m, conversion of 29% of the RNA sample to a more rapidly sedimenting form occurs. The sedimentation pattern for this experiment is shown in Figure V-10(a).

DISCUSSION

An extensive interpretation of the structure of MS2-RNA cannot be made with the present data. We can conclude that the RNA contains no covalent configurational restraints and behaves as a linear molecule upon denaturation. The multiple bands seen under certain conditions are probably due to the hydrogen bonding together of regions of the chain which are significantly separated from one another. That they are not due to mass differences is indicated by the presence of but a single component at higher salt concentrations. The fact that the band pattern can be changed by conditions known to be favorable to the formation or destruction of hydrogen bonds indicates that these bonds do play a role.

The sucrose gradients provide evidence that at least four bands are seen in one RNA preparation. The number



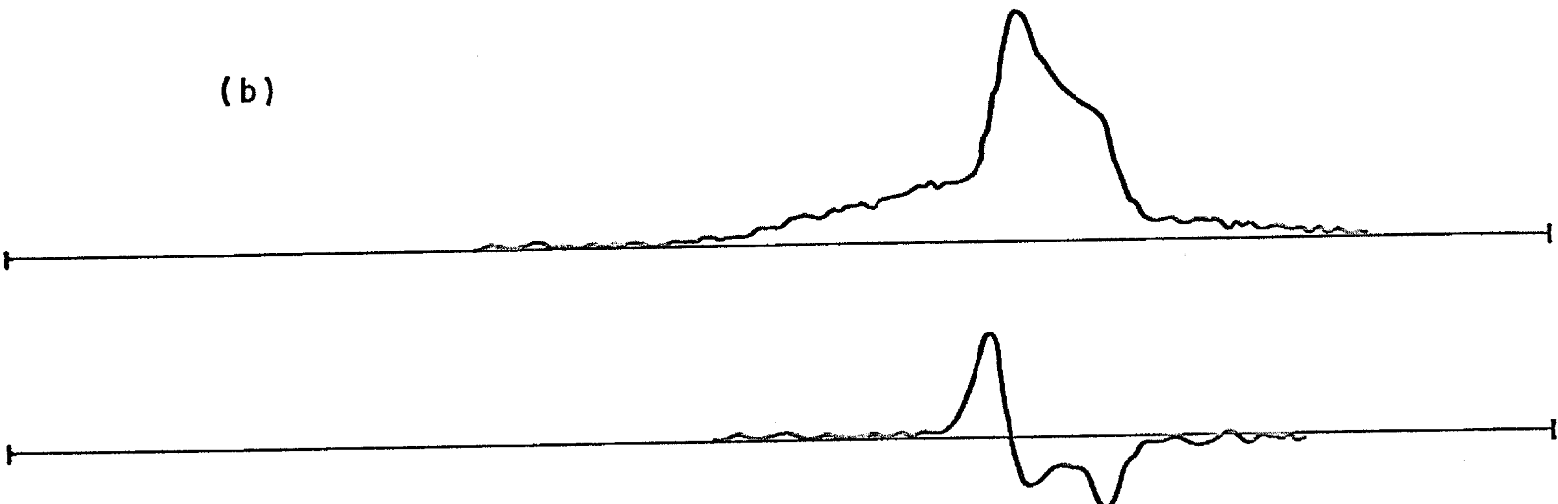


Fig. V-10

Figure V-10 Effect of Heating on MS2-RNA Components

Sedimentation patterns for two of the runs listed in Table V-2 are given. Reading from the meniscus (left to right) a rising boundary is one present in the heated sample but absent (or present in lesser amounts) in the untreated RNA, whereas a falling boundary is one present in the untreated sample but absent in the heated sample. The height of the peak is proportional to the concentration of material of changed sedimentation coefficient. The width of the peak is related to the percent change in sedimentation coefficient.

Sedimentation is in HMP at 25°C.

(a) RNA heated in 0.11 M sodium phosphate pH 7 for 20 minutes 50°C. Photoelectric scan after 28 minutes at 56,100 rpm.

(b) RNA heated in 0.11 M sodium phosphate pH 7 for 3 minutes at 70°C. Photoelectric scan after 24 minutes at 56,100 rpm. Below the optical density tracing is shown the derivative tracing.

of bands seen analytically cannot be accurately assessed because of the scatter of sedimentation coefficients; however, when a heat-treated RNA sample is sedimented simultaneously with an untreated sample at least three boundaries are seen on occasion. The fact that the bands or boundaries are homogeneous leads us to believe that the regions involved in the formation of the configurational restraints are specific and limited in number. The presence of three or four components would require at least four such regions.

Divalent cations appear to play some role in stabilizing the various components under our conditions, as the presence of 0.005 M EDTA results in but a single observable boundary. Although this complicates the interpretation of the results, such a requirement would not be unreasonable. Two competing effects occur in this system. The RNA must be extended so that configurational differences are revealed; at the same time the bonds responsible for the configurational differences must be stable, and they are destroyed by complete denaturation. We might note that in going from an ionic strength of 0.2 to 0.02 at 25°C, the sedimentation coefficient of the RNA is decreased by 30 to 40%, indicating extension of the molecule. The hyperchromicity at 260 mμ is only about 2%, however, indicating that the base stacking is little

affected.

RNA samples dialyzed against 0.01 M EDTA also have their sedimentation coefficient increased by proper heating conditions. If divalent cations play a role in this conversion, it is only what can be scavenged from the 0.11 M phosphate buffer used during heating.

Since all of the components are infective, the possible significance of these forms in viral replication is unknown. The form found in the centrifugal cell will not be the same as found in the infectivity assay, for high Mg^{++} concentrations are involved. Exposure of the RNA to these Mg^{++} concentrations results in a large increase in the sedimentation coefficient. Hydrogen-bonding together of separated parts of the chain could provide the recognition mechanism for the viral RNA-polymerase (27) and could potentially provide other control mechanisms.

It is difficult to estimate the separation of the regions involved in the configurational restraints, for we do not know how the various forms are related. For the two principal bands observed, the ratio of sedimentation coefficients is 1.13 by band centrifugation and 1.08 by boundary centrifugation. For ØX-DNA in band centrifugation, we have obtained a ratio of about 1.10 for the ring and open forms. If the two bands of MS2-RNA are related

by the formation of one set of hydrogen bonds, the regions involved would appear to be widely separated, possibly comprising the ends of the molecule.

The results of Strazielle et al. with turnip yellow mosaic virus (TYMV) may also be explained by such a mechanism. These authors studied the radius of gyration of their RNA preparations as a function of the molecular weight. Molecular weight changes were effected by addition of controlled amounts of EDTA (feasible because their preparations contained hidden breaks stabilized by divalent cations) or by RNase. From the increase in radius of gyration found just prior to decrease of molecular weight they concluded that their preparations consisted of complete or partial rings. Their experimental conditions (HMP, 22°C) are similar to ours, where we have shown the configurational restraints of MS2-RNA to be (partially) stable. Thus hydrogen bonding may be responsible for the formation of rings or partial rings in TYMV-RNA.

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