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*LINCOMYCIN, AN INHIBITOR OF AMINOACYL SRNA BINDING TO RIBOSOMES**

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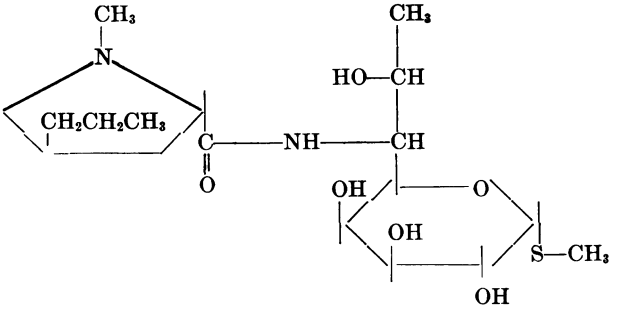
Lincomycin is an antibiotic produced by *Streptomyces lincolnensis* and it has recently been shown to possess the following chemical structure.[1]



Previous studies[2] have shown that lincomycin inhibits protein synthesis in gram-positive bacteria without interfering with DNA and RNA synthesis. This study was initiated to attempt to explain this specificity of lincomycin inhibition and to define the site of action of lincomycin within the over-all protein-synthesizing system.

For this investigation, it was necessary to develop *in vitro* protein-synthesizing systems which utilize combinations of ribosomes, hybrid ribosomes formed by the

reassociation of heterologous 30*S* and 50*S* subunits, and heterologous supernatant. By independently varying species-specific properties of such ribosomal systems, it is possible to determine some features of the mechanism of action of lincomycin and to develop methods which may have general applicability to investigations on the mode of action of other antibiotics and the mechanism of protein synthesis.

Materials and Methods.—Escherichia coli (strain KB, obtained from S. Benzer) was cultivated as described by Lehman *et al.*[3] *Bacillus stearothermophilus* (strain 1503, obtained from E. McCoy), cultivated as described by Friedman and Weinstein,[4] was harvested during the early phase of growth at $OD_{650} = 1$. The culture was rapidly chilled by addition of ice and the cells were harvested with a Sharples centrifuge. The cells were stored as a frozen paste at −20° until used. The pilot plant facility of the Biochemistry Department at the University of Wisconsin (supported by NIH grant FR-00214) was employed for these preparations. We are grateful to J. Garver, A. Olson, and G. Propper for their help with these preparations.

E. coli sRNA was prepared by phenol extractions of intact cells as described by von Ehrenstein and Lipmann.[5] For *B. stearo*, it was found preferable to disrupt the cells first by grinding with levigated alumina (Norton Co., Worcester, Mass.). The supernatant liquid obtained by centrifugation at 100,000 × *g* for 120 min was extracted with phenol. The remainder of the procedure was similar to that used above for the preparation of sRNA from *E. coli*.

The sRNA was charged with C^{14}-phenylalanine as described by Nathans and Lipmann.[6] Ribosomes were prepared by the ammonium sulfate method of Kurland,[7] and the SFII fraction of Wood and Berg[8] or dialyzed S-100 fraction[9] was used as source of supernatant enzymes as indicated.

Amino acid incorporation into protein was performed with slight modifications of the method of Nirenberg and Matthaei.[9] For details, see figures.

Lincomycin was generously provided by G. Savage of the Upjohn Company, Kalamazoo, Mich.

C^{14}-phenylalanine (specific activity 330 μc/μmole) and C^{14}-isoleucine (specific activity 220 μc/μmole) were obtained from the New England Nuclear Corp., Boston, Mass. Poly U was obtained from the Miles Co., Clifton, N.J. Sodium salts of ATP, GTP, PEP were obtained from the Sigma Chemical Co., St. Louis, Mo. Pyruvate kinase (Boehringer) was obtained through Calbiochem, Los Angeles, Calif. C^{14}-poly U was prepared using polynucleotide phosphorylase from *M. lysodeikticus*, as described by Steiner and Beers.[24]

Preparation of 30S and 50S ribosomal subunits: Ribosomes were separated into 30*S* and 50*S* subunits by density gradient centrifugation in sucrose by a modification of the method employed by Gilbert.[11]

The following buffers were used. Buffer A: 10^{-2} *M* Tris-HCl, pH 7.8; 10^{-4} *M* $MgCl_2$; and buffer B: buffer A, containing 5×10^{-2} *M* KCl; 7×10^{-3} *M* β-mercaptoethanol.

The sucrose solution used in preparing the gradient was previously treated with bentonite as follows: 5 gm bentonite (U.S.P. grade, Fisher Scientific Co., no. B-325, used without further purification) was thoroughly dispersed in 1,000 ml of a 20% solution of sucrose containing 10^{-2} *M* Tris-HCl, pH 7.8. Bentonite was removed by centrifugation at 30,000 × *g* for 30 min. $MgCl_2$ was added to a final concentration of 10^{-4} *M*, and the gradient was prepared.

Frozen cell paste was thawed and suspended in buffer A taking 2 ml buffer per gram of paste. Deoxyribonuclease was added to a final concentration of 3 μg/ml. The suspension was disrupted by sonication, and the cell extract was centrifuged twice at 30,000 × *g* for 15 min, each time saving the supernatant. The supernatant was layered on top of a linear sucrose gradient (5–20% in buffer B).

The samples were centrifuged (Spinco rotor SW25, 25,000 rpm, 7 hr, 4°) and fractions containing 10 drops (approximately 0.5 ml) were collected in the usual manner, after piercing the bottoms of the tubes. The ribosomal subunits were stored on ice and used within 48 hr.

Reassociation of ribosomal subunits: For analytical studies, the reaction mixture for reassociation of ribosomal subunits contained: 30*S* subunits, 0.6 OD_{260} units; 50*S* subunits, 1.2 OD_{260} units; Tris-HCl, pH 7.8, 2 μmoles; $MgCl_2$, 2 μmoles in a total volume of 0.2 ml. The sample was incubated on ice for 2 hr. The incubation mixture was diluted to 1.0 ml with Tris-magnesium buffer of the same concentration and analyzed with an analytical ultracentrifuge (Spinco model E). Sedimentation of the boundary was monitored by means of an ultraviolet absorption optical system. The control sample was prepared by pipetting the same quantities of 30*S* and 50*S* sub-

units directly into 1.0 ml Tris-magnesium buffer (same final concentrations) and spinning the sample immediately without prior incubation.

We are grateful to R. Bock, F. Webb, and A. Hempel of the Department of Biochemistry, University of Wisconsin, for their assistance in performing the analytical studies.

Ribosomal binding of aminoacyl sRNA and C^{14}-poly U: The binding of aminoacyl sRNA to the messenger ribosome complex was assayed as described by Leder and Nirenberg.[12] The binding of C^{14}-poly U to ribosomes was assayed according to the method of Spyrides and Lipmann.[10]

Results.—Lincomycin inhibits protein synthesis of gram-positive organisms, whereas gram-negative organisms are not affected.[13, 14] The question arises whether this differential sensitivity reflects an essential difference in some aspect of protein synthesis in these two classes, or whether it is merely due to a difference in relative permeability to the antibiotic. *B. stearo* and *E. coli* were used as representative gram-positive and negative organisms, respectively. It was found that the lincomycin sensitivity of cell-free protein-synthesizing extracts paralleled the sensitivity pattern which was observed in intact cells (Fig. 1). Inhibition of protein synthesis in gram-positive organisms is, therefore, characteristic of the protein-synthesizing apparatus and is not due to relative differences in membrane permeability. A similar inhibition pattern was observed in the presence of lincomycin for C^{14}-isoleucine incorporation in the presence of poly U and at magnesium concentrations ranging from 0.01 to 0.04 *M*.

Next it was important to determine whether sensitivity to lincomycin is associated with the ribosomes or with the soluble factors involved in protein synthesis. Ribosomes from *E. coli* were combined with supernatant enzymes from *B. stearo* and vice versa, and the inhibition of polyphenylalanine synthesis in the presence of 10^{-4} *M* lincomycin was determined. The results of this analysis (Table 1) suggest that the ribosome is the site of inhibition because polyphenylalanine synthesis was inhibited only when ribosomes of *B. stearo* were included in the assay system.

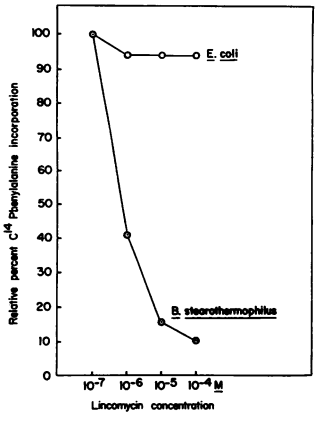


FIG. 1.—Inhibition of protein synthesis *in vitro* as a function of lincomycin concentration. The incubation was performed in a total volume of 0.5 ml and contained (in μmoles unless otherwise noted): Tris HCl, pH 7.8, 50; $MgCl_2$, 7; KCl, 25; β-mercaptoethanol, 3.5; ATP, 0.50; GTP, 0.015; PEP, 2.5; PEPkinase, 20 μg; C^{14}-phe S.A., 330 μc/μmole, 0.1 μc; poly U, 25 μg; iS-30 (*E. coli* or *B. stearo*), 0.8 mg protein; lincomycin, as indicated. The reaction mixture was incubated at 37° for 30 min and assayed for incorporated radioactivity by the hot TCA extraction method. In the absence of lincomycin, the *E. coli* system incorporated 8,260 cpm with poly U and 210 cpm without; the *B. stearo* system incorporated 21,330 cpm with poly U and 1,000 cpm without. Incorporation of C^{14}-phenylalanine in the presence of 10^{-7} *M* lincomycin was 8,530 cpm for *E. coli* and 19,700 cpm for *B. stearo*.

Bacterial ribosomes are composed of two reversibly dissociable subunits. An attempt was made to determine whether lincomycin sensitivity is associated with the 30*S* or the 50*S* subunits alone, or whether lincomycin affects both subunits simultaneously, e.g., their mutual binding. Two approaches were tried. The first one consisted of attempting to recombine subunits from lincomycin-sensitive ribosomes with those obtained from a resistant mutant of *B. stearo*. This approach was unsuccessful because extracts prepared from a resistant mutant were found to

TABLE 1

INHIBITION OF PROTEIN SYNTHESIS BY LINCOMYCIN IN SYSTEMS RECONSTITUTED FROM HOMOLOGOUS AND HETEROLOGOUS RIBOSOMES AND SUPERNATANTS

Ribosomes	Supernatant	C^{14}-Phenylalanine Incorporation (cpm) − Poly U	+ Poly U −lincomycin	+ Poly U + lincomycin 10^{-4} M	% Inhibition
E. coli	*E. coli*	130	3660	5220	0
B. stearo	*B. stearo*	210	3280	490	85
E. coli	*B. stearo*	160	5120	4730	9
B. stearo	*E. coli*	110	1100	256	77
E. coli	None	...	50	...	...
None	*E. coli*	...	92	...	...
B. stearo	None	...	104	...	...
None	*B. stearo*	...	70	...	...

The incubation mixture was the same as that described in Fig. 1, except that ribosomes (10 OD_{260} units), prepared by the method of Kurland,[7] and S-100 protein (0.3 mg) were used instead of the dialyzed iS-30 fraction.

be sensitive. This implied that cellular impermeability was the basis for antibiotic resistance observed in this type of mutant.

A second approach using reassociation of heterologous subunits was therefore attempted. Ribosomes from *E. coli* and *B. stearo* were fractionated into subunits by means of sucrose density gradient centrifugation.

TABLE 2

RECONSTITUTION OF RIBOSOMAL SUBUNITS FROM *E. coli* AND *B. stearo*

Source of 30S subunit	Source of 50S subunit	Incorporation (cpm)
E. coli	—	75
—	*E. coli*	75
E. coli	*E. coli*	2105
B. stearo	—	55
—	*B. stearo*	225
B. stearo	*B. stearo*	800
B. stearo	*E. coli*	1005
E. coli	*B. stearo*	4725

Ribosomal subunits were reassociated as indicated in *Materials and Methods*. Incubations were performed in 0.5 ml and contained (in μmoles unless otherwise noted): Tris-HCl, pH 7.8, 50; $MgCl_2$, 7; KCl, 25; β-mercaptoethanol, 3.5; ATP, 0.5; GTP, 0.015; PEP, 2.5; pyruvate kinase, 20 μg; poly U, 19 μg; C^{14}-phenylalanine, specific activity 330 μc/μmole, 0.1 μc; *E. coli* sRNA, 200 μg; SFII fraction, 100 μg protein; ribosomal subunits as indicated, in OD_{260} units, *B. stearo* 30S, 0.36; *B. stearo* 50S, 0.72; *E. coli* 30S, 0.40; *E. coli* 50S, 0.72. The reaction mixture was assayed by extraction with hot TCA. A reagent blank containing SFII protein incorporated 275 cpm C^{14}-phenylalanine. This value was subtracted from the data.

The complementary subunits were reassociated in four possible combinations as described and these were found to be active in protein synthesis (Table 2). When the products of a reassociation experiment were examined in the ultracentrifuge, a more rapidly sedimenting component was seen which was not present in either of the subunit preparations (Fig. 2).

$S_{20,w}$ values determined for the three components were 29S and 48S for the lighter subunits, and 64S for the heavy component presumably formed by reassociation. Because these values were not considered to be significantly different from the standard 30S, 50S, and 70S values, the latter designations were used in Figure 2. Thus, ribosomal subunits from two different species of bacteria may be reassociated physically and functionally to form active particles. Indeed, the combination *E. coli* 30S plus *B. stearo* 50S has been found in other experiments to be more active than either reconstituted homologous combination. The basis for this relatively higher synthetic activity is not well understood.

Hybrid ribosomes reconstituted from 30S (*E. coli*) and 50S (*B. stearo*) are sensitive to lincomycin, whereas those reconstituted from 30S (*B. stearo*) and 50S (*E. coli*) are resistant (Fig. 3). These data imply that the 50S ribosomal subunit is a sensitive site of lincomycin action.

The 30*S* ribosomal subunit from *E. coli* contains a binding site for messenger RNA and the 50*S* subunit contains at least one binding site for sRNA.[11, 15] It was, therefore, of interest to determine whether lincomycin inhibits the sRNA-binding function of the ribosome. The binding of C^{14}-phenylalanine-sRNA to the ribosome-poly U complex was tested, using ribosomes from *B. stearo.* More pronounced inhibition of sRNA binding to the ribosomes obtained from *B. stearo* was observed (Fig. 4). This is in agreement with the results obtained in incorporation studies. The products of the binding reaction were also examined by sucrose density gradient centrifugation using C^{14}-phenylalanine-sRNA and ribosomes in a homologous system from *B. stearo.* The absence of C^{14}-phenylalanine-sRNA binding in the presence of 10^{-4} *M* lincomycin was also demonstrated by this method.

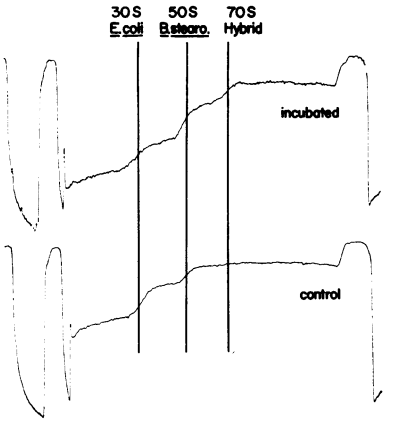


FIG. 2.—Reassociation of 30*S* subunits of *E. coli* with 50*S* subunits from *B. stearo.* The reassociation was performed as described in *Materials and Methods.* The samples were spun at 35,600 rpm at 4°. The tracings shown were taken at 28 min.

In order to determine whether the inhibition produced by lincomycin is specific for the 50*S* subunit or whether it produces a more generalized inhibition of the ribosome, a function of the 30*S* subunit was also tested, viz., the binding of messenger RNA. The binding of C^{14}-poly U to *B. stearo* ribosomes was examined. No decrease was observed in the fast-sedimenting fraction (fractions greater than 70*S*) at 10^{-4} *M* lincomycin—a concentration which inhibits protein synthesis *in vitro* to the extent of 75–80 per cent (Fig. 5).

The above results indicate that the mode of action of lincomycin is the inhibition of the binding of aminoacyl sRNA to the messenger ribosome complex and that a site of action is the 50*S* ribosomal subunit.

Discussion.—Lincomycin belongs to a class of antibiotics which inhibits the binding of sRNA to the ribosome-messenger complex. One of these, chlorotetracycline, which is a broad-spectrum antibiotic, has been shown to inhibit the sRNA-binding step in *E. coli.*[16, 17] Connamacher and Mandel[18] observed the binding of H^3-chlorotetracycline to the 30*S* subunit. In contrast, edeine has also been found to inhibit the sRNA-binding step in *E. coli,* but C^{14}-edeine itself binds only to the 70*S* ribosome of *E. coli.*[19, 20] No data has been obtained yet regarding the ribosomal binding of labeled lincomycin. From our studies it appears, nevertheless, that gram-positive organisms have some features not shared by ribosomes obtained from gram-negative organisms, yeasts, or higher forms.

Species-specific properties of sRNA are probably not involved in the mechanism of lincomycin action since the binding of sRNA from both *E. coli* and *B. stearo* is inhibited only if the ribosomes are obtained from *B. stearo.* This implies that the species-specific aspects of lincomycin action are characteristic of the ribosome exclusively.

The possibility of reassociating heterologous ribosomal subunits was demonstrated by Lederberg and Lederberg.[21] They reported that 70*S* material could be

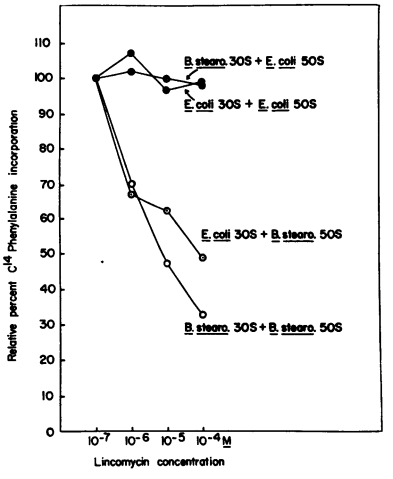


FIG. 3.—Lincomycin sensitivity of polyphenylalanine synthesis in systems reconstructed from hybrid ribosomes which contain heterologous 30*S* and 50*S* subunits. The incubation mixture was the same as that described in Fig. 1, except that 30*S* subunits, 0.6 OD_{260} units, 50*S* subunits, 1.2 OD_{260} units were used as indicated; 100 μg of SFII fraction and 200 μg of *E. coli* sRNA were used in addition. Data pertaining to the synthetic activity of reassociated ribosomes in the absence of lincomycin is presented in Table 2. In the presence of 10^{-7} *M* lincomycin, the incorporation of C^{14}-phe was: *E. coli* 30*S* plus *E. coli* 50*S*, 2,125 cpm; *B. stearo* 30*S* plus *B. stearo* 50*S*, 1,075 cpm; *B. stearo* 30*S* plus *E. coli* 50*S*, 1,385 cpm; *E. coli* 30*S* plus *B. stearo* 50*S*, 7,725 cpm.

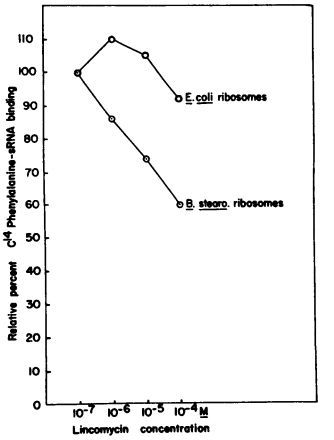


FIG. 4.—Inhibition by lincomycin of C^{14}-phe-sRNA binding to ribosomes. The incubation was performed in a total volume of 0.5 ml and contained (in μ-moles unless otherwise noted): Tris-HCl, pH 7.6, 25; $MgCl_2$, 10; KCl, 50; poly U, 40 μg; *E. coli* or *B. stearo* ribosomes, 8 OD_{260} units; phe-sRNA (*E. coli*), 400 μg containing 1,800 cpm. After incubation at 25° for 15 min, the ribosomes were collected, washed, and counted as described by Leder and Nirenberg.[12]

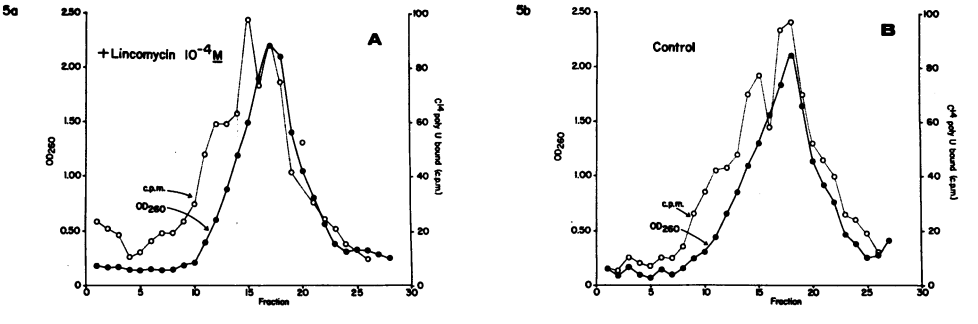


FIG. 5.—Ineffectiveness of lincomycin on C^{14}-poly U binding by *B. stearo* ribosomes. (*A*) *Experimental:* the reaction mixture contained Tris-HCl, pH 7.6, 8.5 μmoles; $MgCl_2$, 1.7 μmoles; KCl, 8.5 μmoles; *B. stearo* ribosomes, 12 OD_{260} units; C^{14}-poly U, 0.3 OD_{260} units; 750 cpm; lincomycin, 0.017 μmole in a total volume of 0.17 ml. After incubation at 30° for 10 min, 0.15 ml of the incubation mixture was applied on top of a 5% sucrose gradient, and the tubes were centrifuged at 36,000 rpm for 90 min in a SW39 rotor. The density gradient contained 10^{-4} *M* lincomycin throughout. (*B*) *Control:* the sample was prepared as above, except that lincomycin was omitted.

obtained by incubating 30*S* subunits from *E. coli* with 50*S* subunits from *Bacillus subtilis* under conditions similar to those described above. The present studies show that such hybrid ribosome preparations are capable of performing protein synthesis.

In the reassociation experiments, it was noted that sensitivity to lincomycin was associated with the 50*S* subunit from *B. stearo.* However, this does not unambiguously prove that only the 50*S* subunit is involved in this inhibition. For instance, lincomycin may act by combining with a receptor site on the 50*S* subunit, other than the sRNA-binding site, producing a structural change in the latter. Indeed, it has been observed that streptomycin can affect the specificity of the binding reaction,[22] whereas sensitivity or resistance to streptomycin is conferred on the ribosome by the 30*S* subunit.[23] Formally, one cannot rule out at this stage a more complicated mechanism of lincomycin action such as a primary effect on the 50*S* ribosomal subunit where it is bound and secondary inhibition of the 30*S* subunit (or vice versa).

The results reported above are not only of interest in elucidating the mode of action of lincomycin, but they also provide technical means of studying ribosome function by taking advantage of characteristic differences between ribosomes obtained from different bacterial species. Heterologous complexes prepared from diverse strains of bacteria which are sensitive or resistant to various chemical agents permit selective control of certain individual processes in the synthesis of proteins.

Summary.—Lincomycin inhibits cell-free protein synthesis in *Bacillus stearothermophilus* but not in *Escherichia coli.* The site of inhibition is the ribosome. The binding of C^{14}-phenylalanine-sRNA but not of poly U to the ribosome is inhibited.

Active ribosomes have been obtained by the reassociation of heterologous 30*S* and 50*S* subunits from the two test organisms, and the use of these ribosomes made possible the identification of the 50*S* subunit as a site of action of this antibiotic. The 50*S* subunit from *Escherichia coli* and the 30*S* subunits of either organism were insensitive to the antibiotic.

These experiments also point out that the 50*S* subunits of the ribosomes from these two organisms have distinctly different properties.

The authors are grateful to C. Harvey, J. Konisky, C. G. Kurland, M. Nomura, and P. Sheldrick for assisting us in this work.

Abbreviations: sRNA, soluble ribonucleic acid; poly U, poly uridylic acid; Tris, tris-(hydroxymethyl)aminomethane; PEP, phosphoenolpyruvate.

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*NUCLEIC ACID OF THE RAUSCHER MOUSE LEUKEMIA VIRUS**

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We wish to report the first successful extraction of an intact RNA from the Rauscher murine leukemia virus, in amounts sufficient for characterization by physicochemical means. As far as we know, this virus contains the largest RNA ever observed. It exhibits a sedimentation coefficient of 73*S* in 0.2 *M* salt, commensurate with a molecular weight of 13×10^6. The virus was obtained from the highest infectious titer source known, the plasma of viremic mice. A preliminary account of these results was given at the Conference on Murine Leukemia, held in October 1965.[1]

Mixed mouse spleen and thymus cell cultures support the growth of virus particles after infection with the Rauscher virus from mouse plasma.[2] A radioactive, RNase-sensitive, 70*S* component was recently detected by rate zonal centrifugation of the extract of fluids from P^{32}-labeled Rauscher virus-infected tissue cultures.[3] Caution must be exercised, however, in equating this RNA, or the RNA obtained from purified Rauscher virus produced in tissue culture, with the RNA of the virus in the plasma of viremic mice. The infectivity of the tissue culture fluids, containing large numbers of virus particles, is 3–5 orders of magnitude below that of the "plasma" virus.[2, 4] The relationship between the particles obtained from an infected tissue culture, and those obtained from the plasma of viremic mice, is not clear. Thus, although we detected RNA more than a year ago as the main nucleic acid component of the purified particles from infected tissue cultures,[5] we decided to concentrate instead upon obtaining an undegraded RNA in good yield from the highly infectious plasma virus.

A suitable method for the extraction of undegraded RNA from animal viruses has long eluded interested investigators. Recently, however, there have been reports