BIOLOGICAL PROPERTIES OF RIBONUCLEIC ACID FROM VIRULENT AND ATTENUATED POLIOVIRUS

By PAUL GERBER, Ph.D., and RUTH KIRSCHSTEIN, M.D.

(From the United States Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health, Division of Biologics Standards Bethesda, Maryland)

Plates 44 to 46

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During the past 2 years the method of Gierer and Schramm (1) for the extraction of infective viral ribonucleic acid (RNA) has been successfully applied to several animal viruses (2–6). The evidence presented by these workers supports the conclusion that the infectivity is due to the RNA preparation itself, and not to residual intact virus particles.

Recent improvements in the preparation and bio-assay of poliovirus RNA reported by Alexander *et al.*, (7, 8) made it possible to examine certain of its biological properties (9, 10). The studies of these investigators (7) demonstrated that the serological type of the viral progeny obtained from RNA-infected HeLa cells corresponded to the type of the RNA donor virus.

The present paper describes the results of a study on neurovirulence of type 1 poliovirus and its relationship to viral RNA. The evidence presented indicates that the genetic information controlling neurovirulence is transmitted by the viral RNA.

Materials and Methods

Virus.—The type I strains of poliovirus used in this study were Mahoney, LSa and LSc. The Mahoney strain is highly virulent in monkeys by the intracerebral and intraspinal route. The 2 variants of Mahoney, LSa and LSc were kindly made available by Dr. C. P. Li. The LSa strain is virulent in mice intraspinally, the LD_{50} being $10^{-2.6}$; the LSc variant is avirulent in mice both intraspinally and intracerebrally and is highly attenuated for monkeys (11).

Cell Cultures.—Monolayers of primary cultures of monkey kidney and rabbit kidney epithelial cells were grown in 2 ounce flat prescription bottles, using medium 199 (12) with 2 or 10 per cent calf serum respectively.

Solutions and Reagents.—Saline A: consists of 0.8 per cent NaCl, 0.04 per cent KCl, 0.035 per cent $NaHCO_3$, and 1 per cent dextrose in distilled water. RNAase:[1] crystalline ribonuclease was dissolved in saline A, 1 mg./ml., sterilized by filtration and stored at −20°C. Agar overlay (13): consists of a mixture of 90 per cent medium 199, 10 per cent skim milk[2] (lab lac),

[1] Obtained from Nutritional Biochemicals Corporation, Cleveland.

[2] Obtained through the courtesy of the Carnation Company, Los Angeles.

0.0017 per cent neutral red and 1.5 per cent Noble's agar. Poliomyelitis immune globulin (human):[3] had a neutralizing titer of 1:800 against the 3 types of poliovirus (100 TCD_{50}).

Virus Titrations.—Plaque-forming units (PFU) were determined on confluent monolayers of primary monkey kidney cell cultures. The growth medium was removed, the cultures were washed with medium 199, drained, and infected with 0.2 ml. of the virus suspension.

After an adsorption period of 1 hour at 36°C. the cells received 5 ml. of the agar overlay. Plaques were counted on the 4th day of incubation at 36°C.

Preparation of Infectious RNA.—The method described by Gierer and Schramm (1) as modified by Alexander *et al.* (7) was used. Virus grown in 32 ounce bottle cultures of monkey kidney cells in medium 199 was sedimented at 105,000 *g* for 3 hours (Spinco, model L No. 40 rotor). The pellet was resuspended in 0.88 M NaCl buffered with 0.04 M sodium phosphate to pH 7.2 using 1/100 of the original volume. The titer of the concentrated virus suspension varied between $10^{8.5}$ and $10^{9.0}$ PFU/0.2 ml.

The RNA extract was adjusted to pH 7.6 with sodium bicarbonate solution and made 1 M with respect to sodium chloride. This preparation is referred to as undiluted RNA. Aliquots sealed in ampules and stored at −70°C. retained their infectivity for at least 8 to 10 weeks.

Infection of Cell Cultures with RNA.—Monolayers of primary cultures of monkey or rabbit kidney cells were washed with 10 ml. of saline A adjusted to pH 7.6, thoroughly drained, and infected with 0.1 ml. RNA, undiluted, or diluted in 1 M NaCl buffered to pH 7.6 with 0.04 M phosphate. After an adsorption period of 30 minutes at room temperature, the cells were washed with 10 ml. of saline A. The monkey kidney cultures received the agar overlay for plaque assay. The RNA-infected rabbit kidney cells were then incubated for 24 hours at 36°C. using 5 ml. of Ginsberg's medium (14) for cell maintenance. Following this step, cells and fluids were frozen and thawed and the supernate was stored at −50°C. for future use. Treatment of RNA with RNAase was carried out by the addition of 0.01 ml. RNAase stock solution to 1 ml. RNA. The mixture was held at room temperature for 5 minutes. Controls received 0.01 ml. saline A per ml. RNA.

Mice.—4- to 5 week-old general purpose random-mated Swiss mice were inoculated by the intraspinal route (15) with 0.2 ml. of virus suspension or RNA. The animals were observed for 14 days for paralysis or death.

Histopathologic Techniques.—The mice were killed with ether. The entire vertebral column was removed as well as the brain, and the tissues were fixed in buffered formol-sublimate for 12 hours after which they were transferred to 10 per cent neutral buffered formaldehyde solution. The vertebral column was divided into 0.5 cm. sections and these were then placed in 5 per cent formic acid for 12 hours for decalcification. Sections were cut 5 μ in thickness and stained with gallocyanin (16).

EXPERIMENTAL RESULTS

In Vitro Experiments.—The studies of Lwoff and Sabin (17, 18) indicated that the reproductive capacity of poliovirus at 39°–40°C. could be correlated with neurovirulence in monkeys. If this property ("t" marker) is determined by genetic factors, it should be demonstrable with infective viral nucleic acids. We compared therefore the "t" markers of Mahoney, LSc and LSa viruses and the corresponding RNA in monkey kidney cultures. The intact viruses were allowed to adsorb for 1 hour at 36°C., the RNA for 30 minutes at room temperature. The results are presented in Table I. The number of plaques formed

[3] Obtained through the courtesy of the American Red Cross.

by the intact Mahoney virus or its RNA was approximately equal at 36°C. and 40°C. On the other hand, no plaques were detectable in the cultures infected with $10^{6.0}$ PFU of LSc virus or its undiluted RNA when incubated at 40°C. The infectivity of RNA was completely abolished following exposure to 10 μg./ml. of RNAase for 5 minutes at room temperature. Similar treatment of RNA with 0.01 ml. saline A/ml. had no effect on the infectivity. Further proof that the genetic character is determined by the viral RNA itself was obtained by "passage" of Mahoney-RNA and LSc-RNA through rabbit

TABLE I

Reproductive Capacity at 36° and 40°C. of Virulent and Attenuated Type 1 Polioviruses and Viral RNA

Inoculum		Virus titers in monkey kidney cultures $\log_{10}$ PFU/0.2 ml. at	
Viral strain	Preparation	36°C.	40°C.
Mahoney	Virus	7.25	7.22
	RNA	2.9	2.88
	RNA, RK passage*	3.7	3.14
	RNA + RNAase	—‡	—
LSc	Virus	7.0	<1.0
	RNA	3.17	—
	RNA, RK passage	2.90	—
	RNA + RNAase	—	—
LSa	Virus	7.2	4.7
	RNA	2.78	1.3

* Virus produced by RNA in rabbit kidney cultures.

‡ No plaques detected with undiluted RNA.

kidney cultures. These cells do not support the multiplication of intact poliovirus but allow the synthesis of complete virus following infection with polio RNA (9). The results indicate that the viral progeny of RNA-infected rabbit kidney cells possessed the same "t" marker, as displayed by the parent, RNA donor, virus. It is of interest that the growth of LSa virus, which is virulent for mice but only partially attenuated for monkeys (11) is partially inhibited at 40°C.

In Vivo Experiments.—Although it was recognized that a study of neurovirulence of poliovirus would be possibly more meaningful in monkeys, nevertheless, for practical reasons, the mouse was selected as a laboratory model. The following series of experiments was performed to compare the multiplication, neurovirulence, and histopathology of intact viruses and the corresponding RNA.

Groups of 31 mice each were inoculated intraspinally with 0.02 ml. of LSc virus ($10^{5.8}$ PFU), LSa virus (10^{4} PFU), or with 0.02 ml. of undiluted RNA. Control groups received LSa-RNA and LSa virus treated for 5 minutes at 25°C. with 10 or 100 μg./ml. RNAase respectively. At 3 and 12 hours, and 1, 2, 3, 5, and 7 days after inoculation, 3 mice of each group were sacrificed, their spinal cords removed, ground, and frozen as 10 per cent suspension in Hanks' balanced salt solution. In addition mice were selected at various intervals for histopathologic examination. The remaining animals were observed daily for 14 days. Occurrence of paralysis and death was recorded. Mice killed or paralyzed during the first 24 hours post inoculation were not included in the calculation of the death rate.

The results shown in Table II represent the average values of 2 separate experiments. As expected, most of the mice inoculated with the virulent LSa strain died or were paralyzed and none of the LSc-inoculated mice showed any

TABLE II

Results of Intraspinal Infection of Mice with Attenuated and Virulent Strains of Type 1 Poliovirus and Their Infective Ribonucleic Acids

Viral strain	Inoculum dilution	$\log_{10}$ PFU/0.02 ml.	Average day of death or paralysis	No. of mice dead or paralyzed
LSc/virus	Undiluted	5.8	—	0/22*
LSc/RNA	Undiluted	1.6	—	0/22
LSa/virus	10^{-4}	4.0	2.4	21/22
LSa/RNA	Undiluted	1.8	2.9	21/22
LSa/virus + RNAase	10^{-2}	4.0	2.5	20/21
LSa/RNA + RNAase	Undiluted	1.8	—	0/22

* Number of mice dead or paralyzed/total number of mice inoculated.

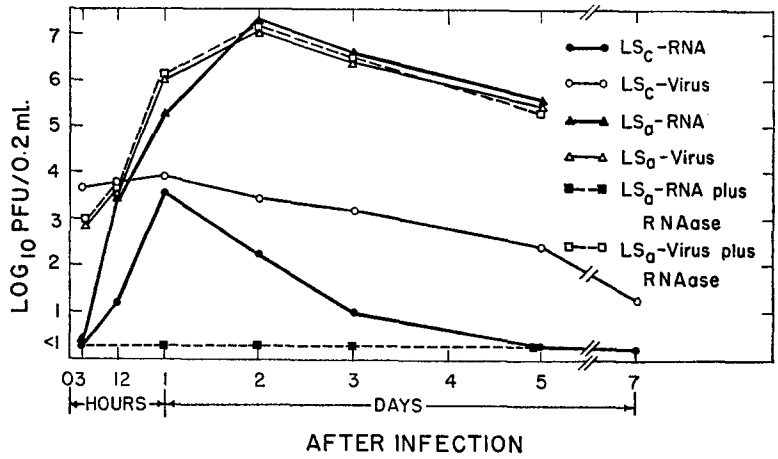
signs of illness. It can be seen that inoculation of viral RNA gave results similar to that of the donor viruses. Exposure of LSa-RNA for 5 minutes at room temperature to 10 μg./ml.RNAase completely abolished its pathogenicity. On the other hand, treatment of LSa virus with 100 μg. RNAase did not influence the outcome of infection.

The virus content of the spinal cords of mice was assayed in monkey kidney monolayers. The results shown in Text-fig. 1 represent average values of 2 separate experiments. The spinal cords of mice inoculated with the avirulent LSc virus contained $10^{3.7}$ PFU /0.2 ml. 3 hours after inoculation. The virus titer did not increase significantly during the first 24 hours and gradually declined within 7 days. The mouse-virulent LSa virus multiplied rapidly during the first 24 hours, reached a peak of $10^{6.8}$ on the 2nd day and maintained a high titer until most of the animals had succumbed. Following inoculation with viral RNA, no virus was detectable at 3 hours. Beginning at 12 hours the growth curves of virus in the RNA-inoculated spinal cords were similar to those of their respective donor viruses. The reason for the fast disappearance of virus in the LSc-RNA group is not known. While RNAase

treatment had no effect on the multiplication of LSa virus, it completely destroyed the infectivity of LSa-RNA

Furthermore, exposure to poliomyelitis immune globulin (final dilution 1:200) for 10 minutes at room temperature had no effect on the infectivity of RNA but neutralized completely the infectivity of intact virus.

The identity of the viruses recovered from the RNA-inoculated spinal cords was established by serum neutralization tests in tissue culture. When used to inoculate mice intraspinally, these agents behaved the same as the respective RNA donor viruses.



TEXT-FIG. 1. Virus content of spinal cords of mice infected with type 1 poliovirus or RNA.

Additional evidence in support of the premise that viral RNA determines the character of virulence was derived from the following experiment. Monolayers of rabbit kidney cell cultures were infected with LSc-RNA or LSa-RNA. When the viral progeny of RNA-infected rabbit kidney cells was inoculated intraspinally ($10^{2.5}$ PFU) into mice, the resulting infections were characteristic of the parent RNA donor virus. Thus, the inheritable character of virulence or attenuation was maintained following "passage" of viral RNA in non-primate cells.

Histopathologic Features.—In all animals, the area of inoculation trauma was seen as a small area of hemorrhage, softening, and light infiltrate of glial cells.

The spinal cords of mice inoculated with LSc virus or LSc-RNA had no lesions even at 5 days (Fig. 1).

Histologically, lesions were found in the spinal cords of mice 2 days after inoculation with LSa virus or LSa-RNA and the lesions increased in severity up to 5 days post inoculation. The first changes seen were those of the motor neurons. There was loss of the coarse Nissl substance characteristic of the mouse motor neurons (Fig. 2). The cytoplasm became glassy. Small infiltrates

of polymorphonuclear leukocytes were seen by 2 to 3 days and these developed around neurons (Fig. 3). Neuronophagia and replacement of neurons by small micro-abscesses were seen by 5 days (Fig. 4). Lymphocytic perivascular cuffing was seen in a few areas.

Figs. 5 and 6 compare motor neurons of mice inoculated with LSc-RNA and LSa-RNA as seen after 5 days.

No lesions of the brains of the mice were seen.

No qualitative or quantitative differences in the development of lesions caused by the intact virus or the infectious RNA were noted. Lesions developed at the same time, to the same degree, and with the same intensity, with the inoculation of either material.

DISCUSSION

The data presented in this study confirm the conclusion reached by others (1–7) that the infectivity of phenol extracts of virus suspensions is associated with RNA. Whether the infective poliovirus RNA is derived from the complete virus particles, from a virus precursor, or both, has not yet been established. The reports by Huppert and Sanders (19) and more recently by Brown and Stewart (20, 21) suggest that in the case of the viruses of murine encephalomyocarditis and foot-and-mouth disease infective RNA may be derived from incomplete virus.

The outcome of *in vitro* and *in vivo* tests for neurovirulence depended on the source of viral RNA. The progeny of Mahoney-RNA showed undiminished reproductive capacity in monkey kidney cell cultures incubated at 40°C. in contrast to RNA extracted from the highly attenuated LSc virus which failed to synthesize complete virus at that temperature. Similarly, intraspinal inoculation of mice with "virulent" RNA caused synthesis of high concentrations of poliovirus in the spinal cords and appearance of typical microscopic lesions, resulting in paralysis and death of the animals. However, when "attenuated" RNA was injected there was relatively low virus multiplication, and an absence of specific lesions, illness, and death.

A single "passage" of poliovirus RNA through primary cultures of rabbit kidney cells, which are insusceptible to intact poliovirus (9) had no effect on the stability of the genetic character of the viral progeny and also furnished additional evidence that the infectivity of the phenol extract could not be ascribed to residual intact virus particles.

The lesions of poliomyelitis in the mouse have been previously described by Eschenbrenner (22). In general, the lesions seen in our mice were similar to those seen by him. The lesions differ from those seen in monkeys in that they are relatively spotty and circumscribed.

The finding that neurovirulence of poliovirus is determined by the viral RNA is in agreement with the observations made by Fraenkel-Conrat (23) in reconstitution experiments with RNA and protein from tobacco mosaic virus.

Since there is apparently no regular correlation between neurotropism for rodents and primates, the observations made in the mouse may not necessarily be extended to primates. It is felt, however, that the mouse serves as an adequate model for the present study.

SUMMARY

Concentrates of type I poliovirus were extracted with phenol by Gierer and Schramm's method. The infectivity of these extracts in tissue culture and mice appeared to be associated with viral RNA.

Studies on neurovirulence based on the "t" marker and intraspinal pathogenicity in mice demonstrated that the viral progeny of RNA isolated from virulent viruses had the "t^+" character and were highly neurotropic for mice. Conversely, RNA extracted from attenuated virus gave rise to a progeny of "t" type which were avirulent for mice.

The results suggest that the infective RNA from type I poliovirus carries the genetic information which determines the degree of neurovirulence.

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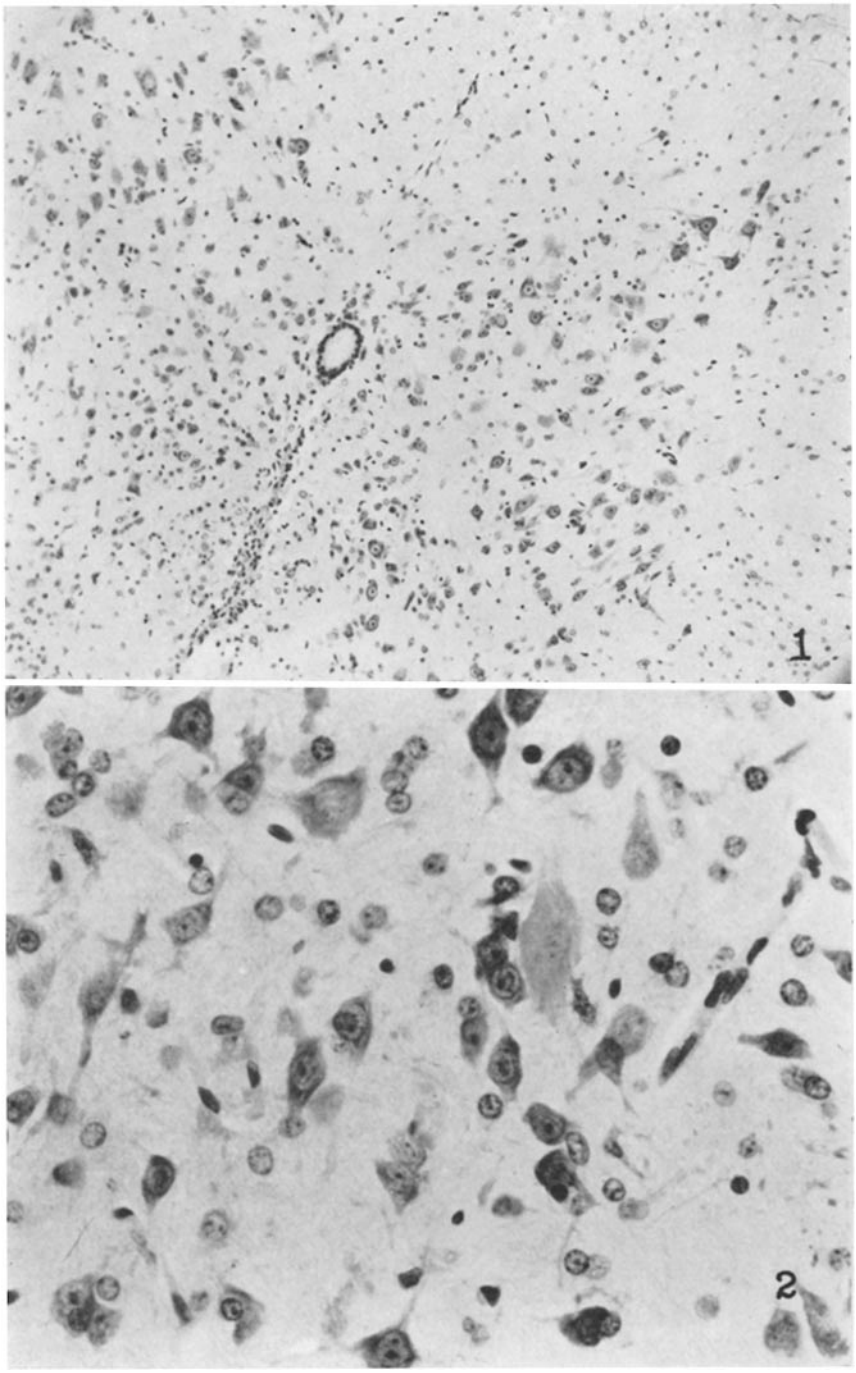
EXPLANATION OF PLATES

The photomicrographs were taken by Mr. John McGuire.

PLATE 44

FIG. 1. Spinal cord of mouse inoculated with RNA from LSc virus 5 days previously. No lesions are seen. Gallocyanin. × 105.

FIG. 2. Spinal cord of mouse inoculated with RNA from LSa virus, 2 days previously. There is marked chromatolysis of several motor neurons and a diffuse infiltrate of inflammatory cells. Gallocyanin. × 415.

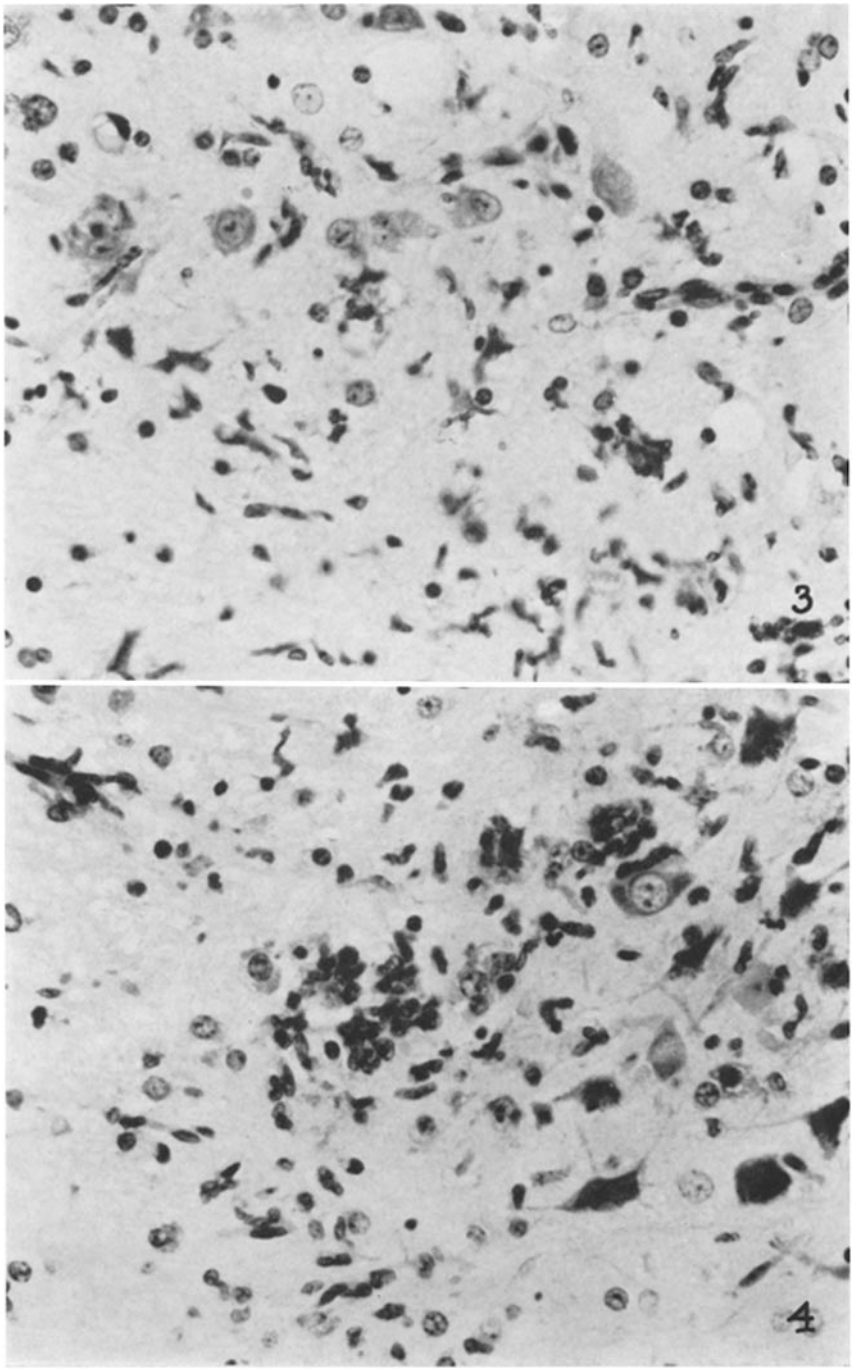


(Gerber and Kirschstein: Biological properties of ribonucleic acid)

PLATE 45

FIG. 3. Spinal cord of mouse inoculated with RNA from LSa virus, 3 days previously. There is chromatolysis of many motor neurons and foci of neuronophagia. Gallocyanin. × 415.

FIG. 4. Spinal cord of mouse inoculated with RNA from LSa virus 5 days previously. Large foci of neuronophagia as well as chromatolytic neurons are seen. Gallocyanin. × 415.

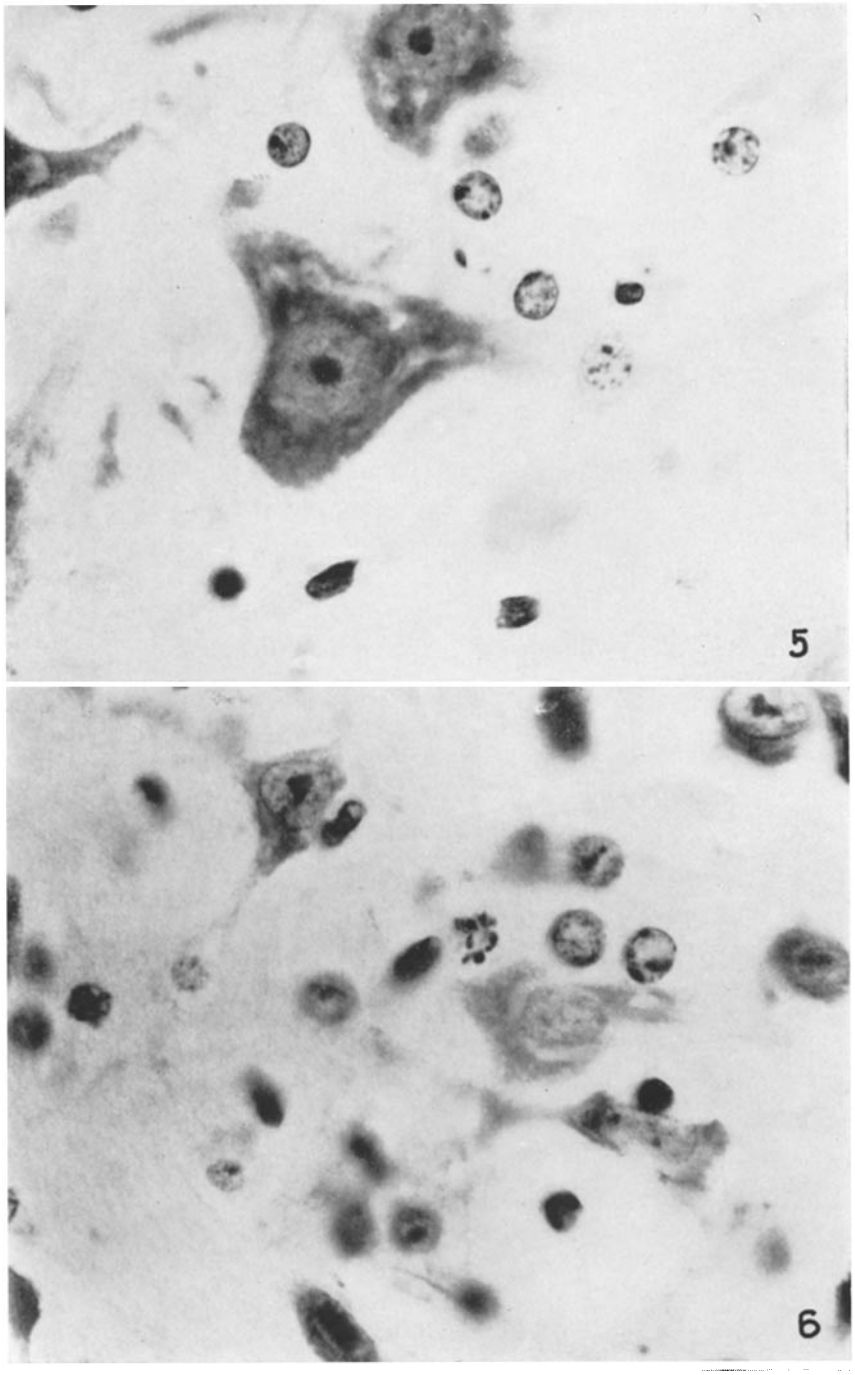


(Gerber and Kirschstein: Biological properties of ribonucleic acid)

PLATE 46

FIG. 5. Normal motor neuron from mouse inoculated with RNA from LSc virus. Note the coarse Nissl substance. Gallocyanin. × 990.

FIG. 6. Marked chromatolysis and early neuronophagia of a motor neuron of a mouse inoculated with RNA from LSa virus. Gallocyanin. × 990.



(Gerber and Kirschstein: Biological properties of ribonucleic acid)