was observed, from the spectrum of each, that at the time required for complete reaction in *n*-propanol (2 hr), the reaction was only about 50 per cent complete in *n*-butanol and in isopropanol, and there was no reaction in methanol or in ethanol. To be especially noted here is that if a small amount of pyridine is added to the ampules containing methanol, ethanol, *n*-butanol, or isopropanol, the reaction does go to completion within 2 hr to form the desired magnesium porphyrin. This, along with the previously mentioned experiments with other solvents, leads us to believe that pyridine exerts a specific effect upon the reaction rather than merely acting as the solvent for the reaction. Work is now being conducted to prove this possibility and to illucidate other phases of the kinetics and mechanism in both model and biological systems.

The authors wish to thank David N. Armentrout for his analysis of our products by flame spectroscopy.

* This investigation was supported by research grant GM 11748 from the National Institutes of Health, USPHS.

† Supported by Molecular Biology training grant 5T1 GM 834 from the National Institutes of Health, USPHS.

[1] Willstaetter, R., and L. Forsén, *Ann. Chem.*, **396**, 180 (1913).

[2] Granick, S., *J. Biol. Chem.*, **173**, 333 (1948).

[3] Wei, P. E., A. H. Corwin, and R. Arellano, *J. Org. Chem.*, **27**, 3344 (1962).

[4] Chu, T. C., and E. Chu, *J. Am. Chem. Soc.*, **74**, 6276 (1952).

---

*INTRODUCTION OF SPECIFIC DRUG RESISTANCE PROPERTIES BY PURIFIED RNA-CONTAINING FRACTIONS FROM PNEUMOCOCCUS**

BY AUDREY H. EVANS†

THE ROCKEFELLER INSTITUTE

Communicated by Rollin D. Hotchkiss, October 16, 1964

The information-bearing function of messenger RNA is supported by two lines of evidence. On the one hand, the relation of certain RNA components of cells to DNA of the same origin is indicated by complementarities in total composition or certain cases of hybrid complex formation which suggest localized complementary arrangements. On the other hand, the correspondence of certain polyribonucleotides with specific peptide structures (indicating "messenger" capacity) is suggested by specific incorporation of particular combinations, or in a few cases of arrangements, of amino acids, when such RNA's are present in biosynthesizing systems.

It seemed important to seek a system in which proteins of specific nature and function might actually be produced as the result of introduction of specific kinds of RNA into cells. Such an experiment could be done in an intact cellular system by introducing RNA of known potentiality into cells not previously containing it. Certain sulfonamide-resistant properties of pneumococcus[1, 2] seemed well suited for this work, since for several reasons they should provide a considerably "ampli-

fied" cellular response from any moderate or temporary messenger activity. Sulfonamide resistance mutation *b* contributes a moderate resistance to sulfanilamide (SA), but a very great resistance toward another p-aminobenzoic acid analogue, p-aminosalicylic acid (PAS). This is manifested in the cell by a folic acid-synthesizing enzyme with modified properties,[3] including the ability to utilize PAS itself,[1, 2] presumably by making a hydroxylated folic acid derivative. Thus, a wild-type cell acquiring RNA having structural information for the *b*-type folic synthetase should show a temporary but relatively sustained resistance to PAS, since there are several levels of amplification: (1) each RNA messenger may lead to formation of a number of specific altered enzyme molecules; (2) each enzyme molecule can (even in the presence of PAS) synthesize many folic acid molecules; (3) folic acid molecules can be accumulated by pneumococcal cells, and in presence of PAS there is probably accumulation of abnormal amounts of the hydroxylated folic acid; (4) each (reduced) folic acid molecule, as coenzyme, can participate in formation of many molecules of each of its catalyzed products (purines, pyrimidines, amino acids, etc.); (5) RNA molecules may well be replicated by cellular enzyme systems.

This article describes the biological activity of RNA-containing material isolated from the *b* and other strains of pneumococcus, resulting in conversion of sensitive cells into resistant ones. The principles outlined above were also applied by the author to detect biological activity of biosynthetically produced RNA in work already published.[4]

Materials and Methods.—The pneumococcal strains used in this work were all derived from the transformable stock R36A, and included two wild-type (i.e., sulfonamide-sensitive) strains, R1 and RUS-7, a *b* donor strain (RUF2S-7), and a *d* recipient strain, RF6-7. The resistance levels for delayed growth of the wild, *b*, and *d* strains, respectively, were: for p-aminosalicylate, 1, 1000, and 0.5 μg/ml, and for sulfanilamide, 4, 16, and 80 μg/ml.[1] Strains carrying the U designation were uracil-requiring, and generally grown with a small quantity of C^{14}-uracil in the medium, which produced a considerable labeling of RNA and a much smaller incorporation in the DNA.

Resistance tests were made as previously described[1] by inoculating 30–100 cells into meat-infusion-neopeptone medium containing antiserum globulin plus various concentrations of the drugs. The number of resistant cells can be reproducibly determined by the number of clones, and the degree of resistance by their size.

Nucleic acid extracts were prepared from cultures in a similar medium not containing antibody, and were purified by the chloroform gel and alcohol precipitation methods used in this laboratory,[5] omitting treatment with RNase but including an exposure to 1 μg/ml of DNase for 15 min at 37°. Following this, a phenol extraction was used to remove protein. Hyperchromic increase of absorption at $\lambda = 260$ mμ resulting from DNase or RNase hydrolysis[5] was used as a measure of the dependence of structure upon DNA and RNA. Denatured DNA was made by heating pneumococcal DNA (prepared with the usual RNase treatment) to 100° for 15 min and quickly cooling. RNA preparations were "annealed" to these by treating briefly with DNase, heating 3–5 min at 100°, then in the presence of an equal amount of denatured DNA in 0.1 *M* sodium citrate–0.15 *M* sodium chloride starting at 50–52° for 30 min followed by cooling to room temperature together during 30 min.

DNase and RNase were Worthington Chemical Co. crystalline preparations of confirmed activity; the latter preparation was heated at 100° for 5 min to destroy any DNase activity.

Results and Discussion.—Induction of drug resistance by RNA preparations: RNA preparations from the *b* strain were tested for their ability to give an early manifestation of PAS resistance in sensitive pneumococci competent for usual DNA transformations. From DNase-treated extracts of pneumococci a number

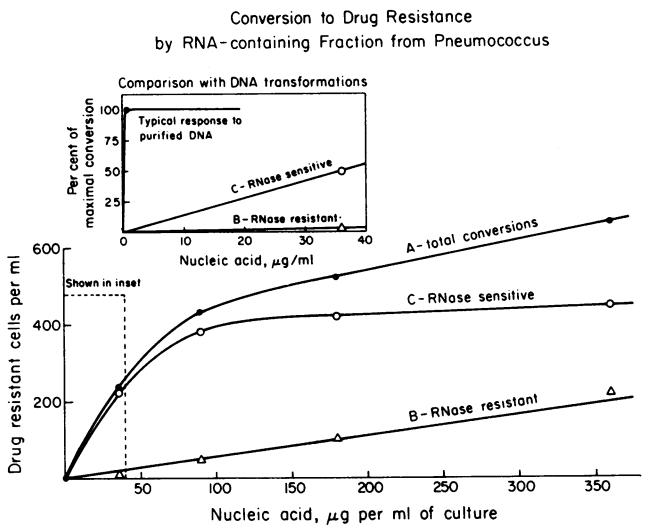

FIG. 1.—Number of p-aminosalicylic acid-resistant transformants, temporary and permanent, produced by exposure of 2.6×10^5 cells per ml to RNA preparation M-4 at concentrations indicated. Drug-resistant cells induced: *A*, total; *B*, after RNase treatment; *C*, RNase-sensitive transformants by difference. Inset graph: low concentration region, in comparison with transformations by purified DNA, typically saturating at 0.1 μg per ml (as percentage of maximal DNase-, or RNase-sensitive conversions).

of nucleic acid preparations were made which showed activity wholly or in part sensitive to RNase. The characteristics of their action upon cells are somewhat different from those of DNA transformations, as shown in Figure 1. Conversion of a population to PAS-resistance reaches a final rate of increase at a nucleic acid concentration of about 100 μg/ml, beyond which point all further increases appear to be due to RNase-insensitive conversions, presumably transformations by residual DNA. The proportion of these latter can vary much more than the RNase-sensitive conversions, among the different preparations. The conversions due only to RNase-sensitive material, curve *C*, reach a plateau value, saturating the cellular response at 100 μg/ml. This is 1000 times less effective than intact DNA, which saturates the standard pneumococcal,[6] *Hemophilus influenzae*,[7] and *Bacillus subtilis*[8] systems characteristically at about 0.1 μg/ml. In fact, the quantity of normal DNA-mediated activity present can be estimated. Thus, the low RNase insensitive activity corresponds to a residual content of only 0.015 μg of intact DNA per 100 μg of nucleic acid, although there could be a larger amount if the DNA is greatly modified or damaged.

Enzyme sensitivity of the active material: As will be seen in Table 1, the biological activity of the RNA preparations is not only RNase-sensitive, but is effectively destroyed by DNase. Since, in preparing the RNA, the nucleic acid fraction is first treated with a low concentration of DNase, this must mean either that at this stage a portion of the DNA escapes hydrolysis, or that although somewhat hydrolyzed, it resists biological inactivation. The RNase- and DNase-sensitivity later manifested by the biologically active material indicates that it is ultimately dependent upon intactness of both RNA and DNA. The reasonable possibility seems to be that it is a complex, such as an RNA-DNA hybrid, perhaps one pre-

TABLE 1

NUCLEASE INACTIVATION OF BIOLOGICAL ACTIVITY OF RNA-CONTAINING FRACTION FROM PNEUMOCOCCUS

RNA preparation		μg/ml	Conversions per 10^5 Recipient Cells		
			Untreated	RNase-treated	DNase-treated
M-1		75	48	5	0
"		150	79	1	0
M-3		45	300	175	10
M-4		36	40	4	2
DNA control		0.3	4400	5200	0
Column Chromatographic Fractions of M-3 and M-4					
M-3 0.5	*N* salt	2.3	69	50	3
0.6	"	2.7	27	12	
0.65	"	2.2	25	32	
0.85	"	2.3	0		
M-4 0.5	*N* salt	1.2	24	0	2
0.6	"	0.5	11	0	0
0.7	"	2.7	9	7	0
0.8	"	1.4	0	0	0

existing as a normal stage of the functioning of DNA in RNA synthesis ("transcription"). It is conceivable that such a hybrid could be relatively resistant to DNase in its natural form, being rendered more susceptible to this enzyme during the isolation process, particularly during phenol extraction, perhaps by removal of residual proteins, such as histones or possibly the polymerase engaged in its formation.

Both RNA and DNA are important for the biological transforming activity of these preparations. If the complex is an RNA-DNA hybrid, it is not composed of two strands of intact DNA and one of inert RNA, for such a complex should be rendered more active by RNase. The hyperchromic increment of absorption of hydrolysis reveals that the major part of the crude material depends for its structure upon RNA and a lesser part upon DNA. The increments at 260 mμ were for preparations M-1, M-2, and M-3, respectively: with DNase 15 per cent, 11 per cent, and 20 per cent; and with RNase 33 per cent, 30 per cent, and 28 per cent. The RNase-resistant transforming activity of M-3 is the highest (Table 1) but only equivalent to a residual content of 0.1 per cent or less of intact DNA. Clearly, the substantial amount of DNA-dependent structure (a 33% increment being a typical maximum for two-stranded structures) which it contains, and the somewhat smaller but significant amounts which the other preparations show, are due to DNA components which must be considerably damaged by chain breaks and are of only low biological activity. Since most of the DNA is also RNase-sensitive, it must be supported, in some hybrid structure, by relatively intact RNA strands. The support which the DNA furnishes to the RNA could endow the hybrid with sufficient structural intactness that it is active like double-stranded DNA,[9] but because of the breakages in the DNA, not enough to give it the RNase-resistance described for other hybrids.[10-12] One may expect that "pure" RNA-DNA hybrid, containing some chain-breaks, would give about a 33 per cent hyperchromic effect with *both* RNase and DNase. The amount of C^{14}-radioactivity rendered acid-soluble from uracil-labeled material is far greater with RNase than with DNase, in keeping with RNase sensitivity and the low rate of entry of uracil into DNA pyrimidines. Since the unfractionated material probably contains ribosomal RNA and other components, it seemed unwise to attempt detailed chemical or physical analyses at this stage.

Purification of the active components: From adsorption on a methylated bovine serum albumin-Kieselguhr column,[13] the RNase-sensitive components of these preparations appear to be eluted at lower sodium chloride concentrations than are intact pneumococcal DNA,[14] or the RNase-resistant residual (DNA) activity of the preparation. The DNA component of M-3 is less damaged than that of M-4, and from the former the column separates a first hybrid fraction fairly resistant to RNase. As indicated in the lower part of Table 1, an activity resembling that of (damaged) DNA was recovered in 0.65–0.7 *M* sodium chloride fractions, and the low salt fractions are more sensitive to both DNase and RNase. The specific activity per μg of nucleic acid is also as much as 20–40 times increased over that of crude material and as little as 0.2 μg shows activity. Further purification and the nature of the active fractions will be the subjects of later investigations.

Artificial hybrid nucleic acid: If the DNA of the complex has been damaged by DNase in the isolation procedure, it should be possible to redistribute the RNA strands upon other homologous DNA not bearing the same marker. When 2.8 μg of RNA preparation M-3 were treated with DNase, it was rendered completely inactive. It was thereupon "annealed" with a slight excess of heat-denatured Rl (wild type) DNA by slow cooling together. The product was again active; 1.3 μg, acting upon each 10^5 recipient cell, was now able to convert 23 cells to PAS resistance, and after RNase treatment this had fallen to 13. Since the active material is also DNase-sensitive, it is clear that an active RNA-DNA complex of some sort had again been created. In several such preparations the reconstituted material has been more resistant to RNase and somewhat more so to DNase, than the original material, perhaps because it now is distributed upon DNA that has not been treated with DNase and is therefore more intact.

Nature of the conversions produced by RNA complexes: The cells which respond to RNA-containing fractions, like the donor cells, show the expected resistance to PAS and SA, but resistance to other drugs has not been detected. Resistance conferred exclusively by RNA would be expected to be temporary, disappearing some time after active messenger was no longer present. What was found in RNA-treated populations were numerous clones bearing temporary resistance or fractional resistance for as many as three to six passages, as well as some which lost it in only a few passages. The persistence may be due to the factors already enumerated; also a small fraction of the clones are spontaneous mutants, which have a different and permanent resistance pattern.

The behavior of a group of PAS-resistant clones obtained from wild-type cells treated with the artificial hybrid mentioned above is represented in Table 2. It will be seen that about one half of the clones showed sensitivity to both drugs, or reduced resistance, within a few passages. What was unexpected was the prolonged persistence of the trait, also the occasional ability to return to partial resistance, i.e., a state in which a fraction of the sensitive population was again resistant. There is no evidence, however, that the cells which have been sensitive for more than a few division cycles have regained resistance, whereas each resistant cell (excepting mutants) seems to have the potential to become sensitive. This capability of being lost and the signs of persistence are suggestive of a particulate existence, and even replication, of some determinant.

When unfractionated RNA-DNA complex is used, some preparations may con-

TABLE 2

LOSS OF DRUG RESISTANCE IN CLONES DURING REPEATED PASSAGE IN NONSELECTIVE MEDIUM

Colony	Resistance: Passage:	Sulfanilamide 1	2	3	3A	4A	7	8	p-Aminosalicylic Acid 1	2	3	3A	4A	7	8
1		R	R	R	R	R			R	R	R	R	R		
2		R	s	p	p	p	p	s	R	p	p	s	s	p	p
3		R	p	p	s	s	s	s	R	p	p	s	s	s	s
4		R	p	p	R	R	p	p	R	p	p	R	p	p	R
5		R	s	p			s	s	R	s	p			p	p
6		R	s	p			s	s	R	s	R			p	p
7		R	R	R	R	R	R	p	R	R	R	R	R	R	R
8		R	s	p	s	s	s	s	R	s	p	s	s	s	p
9		R	s	s	p	s			R	s	s	p	s		
10		R	R	R	R	R			R	R	R	R	R		
11		R	s	R	p	p	p	s	R	p	R	p	s	p	p
12		R	p	R	R	R			R	R	R	R	R		
13		R	p	R	R	p			R	R	R	R	p		
14		R	p	p	p	p	p	p	R	p	p	p	p	p	p
15		R	s	p		p	s	s	R	s	p		s	p	p

R, resistant to screening level of drug, like donor cells (or mutant); p, partial growth of a fraction of population at screening level; s, sensitive to screening and lower levels of drug, like recipient cells. Passages: serial passages in nonselective media (7–10 divisions per passage); 3A and 4A were done after 9 weeks in frozen state.

tain enough DNA to give typical transformants (permanently resistant) as well as mutants, but they also show a proportion of temporary phenotypic transformants.

Introduction of a wild-type property with RNA preparations: To test the specificity of the introduced characters, RNA-containing fractions were prepared from wild-type cells. It was observed that these never produced PAS or SA resistance as did those from type *b* cells. To take advantage of a system less subject to spontaneous mutation, the wild phenotype (resistance to p-nitrobenzoic acid, NOB) was selected for in populations of type *d* after they were treated with the wild-type preparations and chromatographic fractions therefrom. Reconstituted artificial hybrids made from these preparations have not yet been tested. The properties detected are a shift from type *d* (resistance to SA 80 μg/ml; to NOB 0.5 μg/ml) toward the wild type (SA 4 μg/ml; NOB 20 μg/ml). In this system, it is possible to assay both the SA-resistant *d* and the NOB-resistant wild cells in a mixed population.

The phenotypic transformants in this case are temporarily resistant both to NOB and SA, as though each cell contains both types of folic synthetase. Such clones, at first completely free of wild-type cells, within a few passages separate into two distinct types. One is a normal, stable wild type; the other is a "modified *d*" (designated *dm*), resistant to SA, but with a slight resistance to NOB—a type not heretofore seen. In a smaller number of cases, the normal NOB-sensitive *d* is produced again. The highly NOB-resistant transformant therefore can give rise to segregants resembling the donor and recipient strains. The slightly NOB-resistant *dm* also appears to be somewhat unstable, itself passing into wild type and sometimes *d* under conditions not yet understood, though far less readily than the fully resistant temporary transformant. Some of the NOB-resistant transformants are already stabilized as wild or *dm* types during the first selection passage or shortly thereafter. The proportions of these types vary considerably therefore in different experiments: unstable ones from 9 to 12 per cent, semistable *dm* from 25 to 62 per cent, *d* about 10 per cent, wild from 25 to 74 per cent.

It should be emphasized that none whatever of these transformants were produced, if the material was treated with either RNase or DNase. Therefore, al-

though the stable wild type resembles the donor strain, its production and that of the novel *dm* type must be attributed to hybrid material rather than DNA.

Nature of the determinants introduced by RNA-DNA preparations: With the *b* donor material, permanently resistant clones might be attributed to DNA transformation, or mutation, and stress was only placed on those which dropped in resistance. But with the wild-transforming agent, all transformants come from hybrid material and some are seen in process of giving rise to "donor" and "modified recipient" segregants. The presence of both donor and recipient information is manifest in the specific drug resistances of the heterozygotic cells, and they are distributed in the segregants. The information may be brought in on both the RNA and DNA components, or only one of these, which owes its activity to the physical structure supported by both. Thus, RNA may act to preserve activity of DNA, or vice versa. One may expect that one DNA strand may be fragmented on entry, as suggested by the finding of Lacks[15] for double-stranded DNA; or possibly the hybrid enters without incurring new damage. The persistence of the information through many divisions before segregation suggests somewhat uneven replication in a somewhat unstable, perhaps "episomal," form. After segregation, the acquired traits of the stable and semistable transformants are transferable by their DNA. It is probable that donor DNA marker has survived; it is known that localized lesions produced by heat do not prevent genetic incorporation of the adjacent regions.[9, 16]

The unusual survival of the markers in a loosely integrated heterozygotic state suggests that this form and the unintegrated state of other episomal systems might be like the hybrid RNA-DNA form which was introduced. One may suppose that such hybrids can be replicated fairly successfully, as identical or complementary sets, by cellular polymerase systems. This temporarily poised balance can be upset sooner or later by loss or through a delayed transformation by the carried DNA, making a stable homozygote or a more balanced heterozygote. Since the RNA-DNA complexes described above are likely to be of different lengths and intactness, it is not surprising that they give transformants of different stability and behavior. The regaining of partly lost properties in some clones is striking and sweeping enough to suggest infectious transfer within the population; it is planned to investigate further this possibility.

The author wishes to thank Dr. R. D. Hotchkiss for his helpful interest and support during this investigation.

* Supported in part by grant AI-03170 from the National Institutes of Health, USPHS.

† Present address: Department of Biology, University of California, La Jolla.

[1] Hotchkiss, R. D., and A. H. Evans, in *Exchange of Genetic Material: Mechanisms and Consequences*, Cold Spring Harbor Symposia on Quantitative Biology, vol. 23 (1958), p. 85.

[2] Hotchkiss, R. D., and A. H. Evans, *Federation Proc.*, **19**, 912 (1960).

[3] Wolf, B., and R. D. Hotchkiss, *Biochemistry*, **2**, 145 (1963).

[4] Hurwitz, J., A. Evans, C. Babinet, and A. Skalka, in *Synthesis and Structure of Macromolecules*, Cold Spring Harbor Symposia on Quantitative Biology, vol. 28 (1963), pp. 59–65.

[5] Hotchkiss, R. D., in *Methods in Enzymology*, ed. S. P. Colowick and N. O. Kaplan (New York: Academic Press, 1957), vol. 3, pp. 692 and 708.

[6] Hotchkiss, R. D., in *The Chemical Basis of Heredity*, ed. W. D. McElroy and B. Glass (Baltimore: Johns Hopkins Press, 1957), p. 321.

[7] Alexander, H., G. Leidy, and E. Hahn, *J. Exptl. Med.*, **99**, 505 (1954).

[8] Spizizen, J., *Federation Proc.*, **18**, 957 (1959).
[9] Roger, M., and R. D. Hotchkiss, these PROCEEDINGS, **47**, 653 (1961).
[10] Hall, B. D., and S. Spiegelman, these PROCEEDINGS, **47**, 137 (1961).
[11] Schulman, H. M., and D. M. Bonner, these PROCEEDINGS, **48**, 53 (1962).
[12] It should be pointed out that previous indications of RNase resistance of RNA-DNA hybrids are gross measures based upon extreme physical changes such as solubility, density, etc., rather than on sensitive biological test.
[13] Mandell, J. D., and A. D. Hershey, *Anal. Biochem.*, **1**, 66 (1960).
[14] Roger, M., these PROCEEDINGS, **51**, 189 (1964).
[15] Lacks, S., *J. Mol. Biol.*, **5**, 119 (1962).
[16] Lacks, S., and R. D. Hotchkiss, *Biochim. Biophys. Acta*, **39**, 508 (1960).

---

*ELECTRON MICROSCOPE STUDIES ON REOVIRUS RNA**

BY PETER J. GOMATOS AND WALTHER STOECKENIUS

THE ROCKEFELLER INSTITUTE

Communicated by George E. Palade, October 23, 1964

Reoviruses are medium-sized, icosahedral RNA viruses that have a widespread distribution throughout the world. Their RNA content is considerably higher than that of other icosahedral RNA viruses, with the possible exception of wound tumor virus.[1, 2] It has been shown that isolated reovirus RNA consists of complementary strands in a double-helical form.[1, 3] Its secondary structure is similar to that of DNA, but differs from it in the number of residues per turn, the distance between base pairs, and in certain other respects.[3] All attempts to isolate the nucleic acid of reovirus in an infective form have so far failed.

This paper describes some of the physical properties of isolated reovirus RNA as observed with the electron microscope and correlates them with the other available physicochemical data.

Materials and Methods.—Reovirus 3, Dearing strain, was grown and purified as described before. The virus preparations, banded in a cesium chloride density gradient, were dialyzed extensively against 0.15 *M* potassium chloride–0.015 *M* potassium citrate. Unless otherwise stated, the nucleic acid of the virus was extracted with phenol by gentle rocking at room temperature. The preparative procedures and the electron microscopy were usually performed immediately after extraction of the RNA. In a few instances, extracted RNA was kept at 4° for one or several days, and the experiment repeated. No differences were noted.

For electron microscopy the RNA was spread in a protein film on a water surface following the procedure of Kleinschmidt *et al.*[4] with slight modifications as described before.[5] In a typical experiment 0.1–0.2 μg viral RNA and 100 μg diisopropylphosphoryl trypsin (DIP-trypsin) were used. Intact virus particles were suspended in 0.1 ml of 1.0 *M* ammonium acetate, 1.0 *M* $CaCl_2$, or 1.0 *M* $MgCl_2$, or 1.0 *M* $MgCl_2$ containing 100 μg DIP-trypsin; 10.9 ml of 7.0 *M* ammonium acetate, or saturated $CaCl_2$, or saturated $MgCl_2$, each containing 4.5% isopropanol, was added a few minutes before the material was spread on 0.015 *M* ammonium acetate. This resulted in release of the RNA from the virus during spreading of the surface film. In a control experiment purified reovirus labeled in its RNA with P^{32} was exposed to 7.0 *M* ammonium acetate containing 4.5% isopropanol and spun at 105,000 *g* for 2 hr in a Spinco preparative centrifuge. Over 95% of the radioactivity was recovered in the pellet, indicating that less than 5% of the virus preparation could have been disrupted by suspension in 7.0 *M* ammonium acetate.

In the preparations for electron microscopy, the ratio of RNA or virus to basic protein was kept low so that the RNA molecules and virus particles were well separated in the film. This is