Demonstration of Sequence Differences in the Ribonucleic Acids of Bacteriophage MS2, and a Mutant of MS2*

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ABSTRACT: Procedures for the detection of small differences in ribonucleic acids of large molecular weight have been developed. The analyses of the oligonucleotides produced by specific enzymic degradation of the ribonucleic acids of the bacteriophage MS2 and a nitrous acid mutant of MS2 have been used to demonstrate sequence differences in these macromolecules. The oligonucleotides were fractionated initially according to size on a DEAE-Sephadex column at neutral pH in the presence of 7 M urea. Subsequently, each group of larger oligonucleotides was subfractionated according to the base content of its components, using a new application of DEAE-Sephadex columns: the ion-exchange chromatography of oligonucleotides at low pH in the absence of urea. Elution patterns obtained for the components of the two ribonucleic acids showed a small number of differences, the most notable of which occurred in the chromatographic group corresponding to the tridecanucleotides. In the case of the wild-type nucleic acid, this group, on subfractionation, produced four oligonucleotide peaks, while in the subfractionation of the corresponding group from the mutant, two of these peaks were shown to be absent. The most reasonable explanation for these two differences observed in the digest of the mutant ribonucleic acid is that they have resulted from sequence changes in the mutant nucleic acid caused by the replacement of two adenine bases in the wild-type nucleic acid by two guanine bases.

In one of the central concepts of molecular genetics, a mutation in a particular organism is considered to arise from a change in the nucleotide sequence of the genetic material (DNA or RNA) of that organism. In its simplest form then, a mutation could result from the substitution of one nucleotide base for another within the linear polynucleotide structure of the organism's nucleic acid. The difficulties to be expected in attempting to detect such sequence changes by chemical means result from two main factors: the large size of those DNA and RNA molecules which carry genetic information, and the availability of only a limited number of methods which might be used for the characterization of their nucleotide sequences. However, the present work shows that progress in the development of methods for the column fractionation of oligonucleotides has made it possible to demonstrate small differences between closely related nucleic acids, and that single base changes within the genetic material of RNA-containing bacteriophages may be detected. Some of the results of these studies have been published in abstract form (Robinson and Gilham, 1967).

The new methods of fractionation stem from the observation that mixtures of oligonucleotides (of size up to the heptanucleotide level) could be separated, essentially in accordance with their chain length, by chromatography on DEAE-cellulose in the presence of 7 M urea (Tomlinson and Tener, 1963). The application of this method to the fractionation of the pancreatic ribonuclease digest of the high molecular weight RNA from a plant virus gave a resolution of oligonucleotides of all sizes with the largest occurring at the undecanucleotide level (Gilham and Robinson, 1964), and improved resolutions of ribonuclease T_1 digests of RNA have been obtained by the use of DEAE-Sephadex in place of DEAE-cellulose in the chromatography of oligonucleotides (Rushizky *et al.*, 1964). Methods by which oligonucleotides of the same chain length may be subfractionated according to their base composition have also been developed (Rushizky *et al.*, 1964; Gilham and Robinson, 1964; Solymosy *et al.*, 1965).

The ease with which a single base change in a mutant RNA molecule might be detected should depend upon the type of mutation that has occurred and upon the nucleotide sequence of the polynucleotide chain within the immediate vicinity of the point of mutation. The replacement of a guanine residue by any other base would be detected by a change in the distribution of groups of oligonucleotides of equal chain length in the fractionation of the ribonuclease T_1 digest of the RNA. The change would involve the disappearance of two oligonucleotides from their respective elution positions and the formation of a new oligonucleotide species whose chain length would be the sum of those of the two missing species. Conversely, the replacement of any other base by a guanine would result in the disappearance of an oligonucleotide with the formation of two smaller species. Although other changes such as pyrimidine to pyrimidine changes would not be detected by alterations in the distributions of groups of oligonucleotides of equal chain length from the ribonuclease T_1 digest, these replacements would become apparent when the appropriate group of oligonucleotides was subfractionated

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TABLE I: Serial Induction of MS2 *ts*3 by Nitrous Acid.

Treatment			Isolation		
Mutant Treated	Fraction Surviving[a]	Fraction of Survivors That Are Temp Sensitive[b]	Mutant Isolated[c]	Highest Temp at Which Mutant Grows[d] (°C)	Reversion Frequency[e]
*ts*0[f]	10^{-7}	0.15	*ts*1	43	10^{-6}
*ts*1	10^{-5}	0.25	*ts*2	42	10^{-8}
*ts*2	10^{-4}	0.20	*ts*3	41	10^{-5}

[a] Assayed on *E. coli* K10 at 41°. [b] Plaques were picked and the fraction unable to grow at the highest temperature at which the parent can grow on an agar plate seeded with K10 was determined. [c] Selected from many candidates by its ability to yield relatively high-titer lysates. [d] Rounded off to the highest integer. [e] Fraction of phage in a lysate that could grow at a temperature 1° higher. [f] Grows at 44.5°.

according to the base content of its components. Thus, for the production of a mutant which could be readily analyzed by ribonuclease T_1 digestion, the most desirable mutagen would be one capable of producing or eliminating guanosine. Nitrous acid treatment was therefore used since it has been shown that this mutagen is capable of inducing both the transitions A→G and G→A in single-stranded nucleic acid (Tessman *et al.*, 1964).

Experimental Procedures

Growth and Assay of Virus. The MS2 bacteriophage and its mutant were grown and assayed on *Escherichia coli* C3000 by the methods described by Davis and Sinsheimer (1963) except that the infection with the virus was carried out with a multiplicity of 0.1 when the bacterial concentration was 2×10^8/ml and the virus was harvested 5–6 hr after infection.

Formation of MS2 Mutants. For the nitrous acid mutagenesis the reaction mixture consisted of 0.2 M sodium acetate buffer (pH 4.0)–1 M potassium nitrite–phage lysate (10:1:5, v/v) (Tessman, 1959). The virus was treated for 6–8 hr at 25° and the reaction was then terminated by a 50-fold dilution into cold 0.1 M Tris-Cl buffer (pH 8.5). Initially, a spontaneously produced mutant, *ts*0, was selected for its ability to grow on *E. coli* K10 (λ^-, trp^-) at 44.5°, which is about 1° higher than the temperature at which the wild type can grow. This mutant was then used as the starting point for the production of mutants unable to grow at successively lower temperatures (Table I). The final mutant, *ts*3, differs from the wild-type MS2 by four mutational steps, one spontaneous and three nitrous acid induced, but from the frequency of temperature-sensitive mutants found at each of the induced steps, it is likely that *ts*3 contains more than four mutations.

Purification of Virus. METHOD 1. $(NH_4)_2SO_4$ (3.6 kg) was added with stirring to the lysate (12 l.; viable titer, 2×10^{12}/ml or higher) and after the salt had dissolved the precipitate was allowed to settle overnight at 5°. The supernatant was siphoned and the remaining sediment was collected by low-speed centrifugation. The pellet was resuspended in about ten volumes of water and the pH of the mixture was adjusted to 8 with concentrated NaOH. Deoxyribonuclease I (2 mg) and lysozyme (200 mg) were added and the mixture was stirred for 30 min at 37°. All subsequent steps in the purification were carried out at 5°. The insoluble material was removed by centrifugation at 15,000*g* for 1 hr and the supernatant was made 2 M in $(NH_4)_2SO_4$. The resulting precipitate was collected by centrifugation (15,000*g*, 30 min) and resuspended in water (600–800 ml), and the mixture was centrifuged at 10,000*g* for 15 min. The virus was obtained from the supernatant by centrifugation at 105,000*g* for 2 hr.

METHOD 2. In more recent experiments a less tedious procedure was used. This procedure involved a modification of the method described by Rushizky *et al.* (1965). The 12-l. lysate was cooled to 5° and all subsequent operations were carried out at this temperature. Chloroform (80 ml) and EDTA (free-acid form, 21 g) were added and, after the mixture had been stirred for 15 min, $(NH_4)_2SO_4$ (3.6 kg) was added and the stirring was continued for a further 30 min. The mixture was allowed to stand overnight and then passed through a Sharples centrifuge (type TIP), operating at 40,000 rpm. The pellet was dispersed in water (750 ml) and stirred for 2 hr, and the aqueous extract was finally centrifuged at 10,000*g* for 30 min. The extraction of the pellet was repeated three times with 500, 400, and then 350 ml of water and the combined supernatants were mixed with $(NH_4)_2SO_4$ (600 g). After being stirred for 2 hr the resulting precipitate was collected by centrifugation at 11,000*g* for 30 min. The pellet was suspended in 0.001 M EDTA buffer (pH 6.1), (200 ml) and dialyzed against the same buffer solution (two 6-l. portions) for 36 hr. The extract was then centrifuged at 11,000*g* for 30 min and the resulting supernatant was centrifuged at 105,000-*g* for 3 hr to give the clear phage pellet. The yields of virus per 12 l. of lysate, employing either method of purification, were in the range 250–750 mg. The yields were determined spectrophotometrically using the value 8.2 ODU_{260}/mg previously determined for the serologically

related phage R17 (Enger *et al.*, 1963). The MS2 phage and its mutant each gave a single peak (s_{20} = 75 S in 0.05 M sodium phosphate buffer, pH 7.0) when examined in the ultracentrifuge with schlieren optics. The ultraviolet spectrum of each phage had an A_{260}/A_{280} of 1.7.

Preparation of Ribonucleic Acid. A suspension of the virus (150–300 mg) in water (6 ml) was added to a mixture of 10 ml of redistilled phenol (saturated with water at 5°), bentonite (prepared by the method of Fraenkel-Conrat *et al.* (1961), 90–150 mg in 3 ml of 0.01 M sodium acetate), 0.01 M EDTA (pH 7) (0.1 ml), and 4 M sodium acetate (pH 6), (0.25 ml). The mixture was shaken for 5 min on a Vortex mixer and then centrifuged to break the emulsion. The water-rich layer was removed and extracted with another 10 ml of liquid phenol. The mixture was shaken and the water-rich layer removed as before. The phenol-rich layer from the first extraction was reextracted with a mixture of 0.1 M EDTA (pH 7), (0.1 ml), 4 M sodium acetate (pH 6), (0.25 ml), and water (10 ml) and the water-rich layer from this mixture was used to extract the phenol-rich layer from the second extraction. Finally, the two water-rich layers were combined and washed repeatedly with ether to remove dissolved phenol. The solution was then centrifuged at 105,000*g* for 1 hr at 5° to remove bentonite. To the supernatant was added 4 M sodium acetate (pH 6) (0.5 ml) and ethanol (40 ml) and the mixture was allowed to stand for a few hours at 0°. The precipitated ribonucleic acid was collected by centrifugation, washed twice with cold ethanol, and dried *in vacuo* to a white powder (yield 60–85% assuming that the virus contains 34% RNA). The product contained 21.2 ODU_{260}/mg and was stable in water at 25° for at least 24 hr as judged by the schlieren pattern of its sedimentation in the ultracentrifuge. Ultracentrifugation with the use of absorption optics showed that all of the product was of high molecular weight and that more than 50% of the RNA sedimented as a sharp boundary (s_{20} = 23.0 S in 0.05 M sodium phosphate buffer, pH 7.0) with the remainder of the material trailing just behind this boundary. The base compositions of the viral RNAs were determined by the method of Katz and Comb (1963): adenosine, 0.22; uridine, 0.26; cytidine, 0.25; and guanosine, 0.27; and these values are identical with those obtained previously for MS2 RNA (Strauss and Sinsheimer, 1963).

Ribonuclease T_1 Digestion of Viral RNA and Fractionation of Products. The viral RNA (75 mg) was dissolved in water (5 ml) and treated with 1.5 mg of ribonuclease T_1 (Sankyo Co. Ltd., Tokyo) and anhydrous disodium phosphate (25.4 mg calculated to produce a pH of 7.5 at the completion of the digestion). A few drops of toluene were added and the mixture was kept at 20° for 20 hr. The solution was made 7 M in urea and then applied to a DEAE-Sephadex column and fractionated by a modification of the technique described by Rushizky *et al.* (1964). DEAE-Sephadex (A-25, medium, 3.5 ± 0.5 mequiv/g) was suspended in 1 M sodium acetate and allowed to swell overnight. The fine particles were removed by repeated mixing with 1 M sodium acetate and decantation. The remaining Sephadex was then suspended in solution A (0.85 M sodium acetate–0.05 M Tris-acetate–7 M urea solution, the pH of which had been adjusted such that it gave a pH meter reading of 7.5) and packed into a column with this solution such that the final dimensions were 2 × 25 cm. The column was then washed with solution B (0.15 M sodium acetate–0.05 M Tris-acetate–7 M urea solution, the pH of which had been adjusted such that it gave a pH meter reading of 7.5) and the ribonuclease digest (obtained from 65 to 75 mg of viral RNA) was applied. The fractionation was carried out at a flow rate of 20 ml/hr with a linear gradient of sodium acetate made from 3.5 l. of solution B and 3.5 l. of solution A. The optical density at 260 mμ of each fraction (*ca.* 10 ml) was determined, and appropriate fractions were combined and dialyzed against water (five 6-l. portions) at 5° for a total of 10 hr. The combined fractions were then concentrated *in vacuo* to a small volume and again dialyzed for 5 hr against water (two 6-l. portions), and finally stored at −20°.

Subfractionation of Oligonucleotide Peaks. DEAE-Sephadex (A-25, medium, 3.5 ± 0.5 mequiv/g) was suspended in 1 M NaCl and after standing overnight the supernatant solution was removed by decantation. In order to remove fine particles the material was repeatedly washed by decantation with the limit buffer of the intended separation. The column (1 × 18 cm) was packed with the limit buffer and then washed with the appropriate starting buffer. The dialyzed aqueous solution of oligonucleotides was applied to the column and the fractionation was carried out with a solution of 0.05 M sodium citrate (pH 2.7) containing an appropriate linear gradient of sodium chloride. Fractions of 1.0–1.6 ml were collected at 20-min intervals and the elution pattern was obtained by the measurement of optical density at 260 mμ.

Results

The elution patterns obtained from the fractionation of ribonuclease T_1 digestions of the MS2 RNA, carried out in duplicate, were essentially indistinguishable from one another, and one of these is shown in Figure 1A.

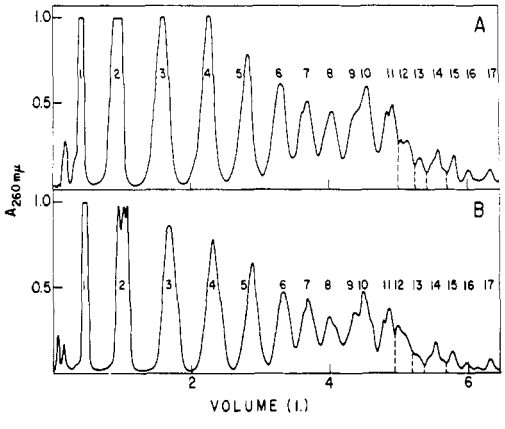

FIGURE 1: Fractionation of ribonuclease T_1 digests of MS2 RNA (A) and MS2-*ts*3 RNA (B). Each elution pattern was obtained by chromatography at 20° on a DEAE-Sephadex column (2 × 25 cm) with 7 l. of 7 M urea–0.05 M Tris-acetate solution (pH meter reading, 7.5) containing a linear gradient of 0.15–0.85 M sodium acetate at a flow rate of 20 ml/hr.

Duplicate ribonuclease digests of the RNA of the mutant, *ts*3 also gave identical patterns and one of these is shown in Figure 1B. The patterns from the parental and mutant RNA appear to be similar except for the amount of material in peak 13. Peak 1 in each case has been identified as guanosine 3′-phosphate and, in view of previous experiences with columns of this type, it seems likely that the majority of oligonucleotides in each of the other peaks are of the same chain length (*i.e.*, a chain length equal to the number assigned to each peak). These assignments are supported by the observation that a plot of the peak numbers versus the volumes required to elute the highest points of the peaks produces a smooth curve. However, it is also clear that the peaks of larger oligonucleotides are not composed exclusively of oligonucleotides of equal chain length since the elution pattern displays considerable overlap between these peaks. Peak 17 represents the largest oligonucleotide obtained in each case since no further material could be eluted from the columns after this point.

The material in each peak was combined and the peaks were separated by making cuts at the lowest points in the valleys of the elution patterns (as shown for some of the peaks in Figure 1). However, the oligonucleotides in peaks 9 and 10 were collected together to avoid the problem caused by the poor resolution of these two peaks. In order to detect, in the primary fractionations, any differences in the oligonucleotides produced by the parental and mutant phages it was considered advantageous to obtain, initially, some estimate of the number of species occurring in each group of oligonucleotides. The measurements were made spectrophotometrically and the calculations were based on an estimate of 15% for the increase in the optical density at 260 mμ exhibited by the two ribonucleic acids on digestion with ribonuclease T_1. Assuming that the absorption at 260 mμ of a base in an oligonucleotide of the size occurring in peak 7 or larger is the same as the absorption of the same base in the intact RNA molecule itself, and assuming that the chain lengths of the ribonucleic acids of MS2 and its mutant are 3340 (the chain length calculated for the related phage, R17; Sinha *et al.*, 1965), the ODU_{260} per average nucleotide in an oligonucleotide of chain length 7 or larger is given by

$$\frac{\text{total } ODU_{260} \text{ after ribonuclease hydrolysis}}{3340 \times 1.15}$$

for a particular digestion. The number of ODU_{260} expected for each oligonucleotide is then obtained by multiplying this value by the presumed chain length of the oligonucleotide. The number of oligonucleotide species present in each peak can be calculated by dividing this number into the total ODU_{260} contained in the peak (Table II). Since the four nucleotides have widely different absorptivities at 260 mμ the calculation used here can give only rough estimates of the number of species. However, the comparison of the values obtained for the wild-type phage with those of the mutant should be significant and it will be seen that some differences are indicated in the amounts of oligonucleotides calculated for the peaks 7, 8, and 13.

TABLE II: Number of Oligonucleotide Species in Peaks from Primary Fractionations.

Peak	Calcd No. of Oligonucleotide Species	
	MS2	MS2-*ts*3
7	34.0	37.0
8	27.5	25.0
9 + 10	41.5	42.0
11	19.5	20.5
12	11.0	10.0
13	3.8	2.5
14	5.4	5.7
15	2.5	2.6
16	0.9	1.2
17	0.9	0.9

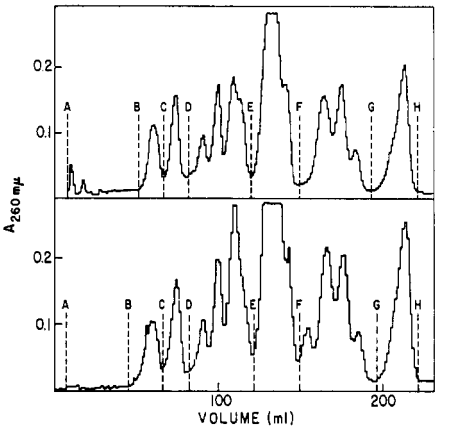

FIGURE 2: Subfractionation of peak 7 from the primary fractionation of the digest from MS2 RNA (upper pattern) and MS2-*ts*3 RNA. Each elution pattern was obtained by chromatography at 20° on a DEAE-Sephadex column (1 × 18 cm) with 400 ml of 0.05 M sodium citrate (pH 2.7) containing a linear gradient of 0.1–0.6 M NaCl at a flow rate of 4.5 ml/hr.

These differences have been confirmed by the subfractionation of the groups of oligonucleotides. For these secondary separations a number of different methods were explored, including the use of DEAE-Sephadex and 7 M urea at low pH (Rushizky *et al.*, 1964). However, a new technique, employing chromatography on DEAE-Sephadex at pH 2.7 in the absence of urea was found to be the most useful. This method gave elution patterns with superior resolution of the components of the oligonucleotide peaks. All the peaks, 7–15, have been subfractionated using this technique and the elution patterns obtained with the peaks 9 + 10, 11, 12, 14, and 15 from the wild-type RNA were found to be essentially superimposable on those obtained with the corresponding peaks from the mutant RNA. On the other hand, the subfractionations of the peaks 7, 8, and 13 showed distinct differences between the products of the two ribonucleic acids.

Two examples are given here to illustrate the sensitivity of the method. Figure 2 shows the elution patterns

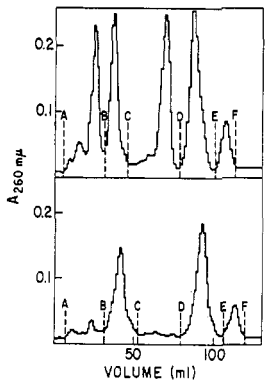

FIGURE 3: Subfractionation of peak 13 from the primary fractionation of the digest from MS2 RNA (upper pattern) and MS2-*ts*3 RNA. Each elution pattern was obtained by chromatography at 20° on a DEAE-Sephadex column (1 × 18 cm) with 400 ml of 0.05 M sodium citrate (pH 2.7) containing a linear gradient of 0.25–0.75 M NaCl at a flow rate of 4.5 ml/hr.

obtained for the peak 7 in each case. It will be seen that they are identical except that the mutant pattern shows an extra oligonucleotide peak in the area, FG. The amount of material contained in this new peak corresponds roughly to that expected for a single species of heptanucleotide. A similar situation was found in the comparison of the elution patterns from peak 8 except that the mutant pattern contained one less peak and again, the amount of material involved corresponded to a single species of octanucleotide. A more dramatic difference was exhibited in the examination of the components present in each peak 13 (Figure 3). The elution pattern for the wild-type products clearly shows the existence of four peaks, each consisting of about the same number of optical density units, while the pattern obtained for the mutant indicates the presence of only two. From their elution positions the two mutant oligonucleotides are presumed to be identical with those in the second and fourth peaks appearing in the wild-type pattern. It was important, at this stage, to prove that these differences did not arise by variation in the positions at which the cuts were made. This was done by a careful comparison of the components of the peaks on either side of peak 13 in each case. The subfractionation patterns for peaks 12 and 14 showed eight and five resolved peaks, respectively, and the MS2 patterns were identical with those of the mutant.

Each low pH separation of peak 13 shows also a small peak appearing in the area EF. The amount of material contained in each of these peaks is considerably less than that required for a single species of a tridecanucleotide. It seems likely that this small peak arises from the overlapping of peaks in the primary fractionation and thus, it would be expected that the remaining portion of this oligonucleotide should appear, in each case, in either peak 12 or peak 14. Indeed, the patterns obtained for both peaks 12 and 14 show peaks with reduced quantities of material in the areas corresponding to EF and it is presumed that one of these represents the other portion of the oligonucleotide appearing as the small peak shown in Figure 3.

Discussion

The present work demonstrates that, with the use of an appropriate specific ribonuclease together with two different ion-exchange separation procedures, it is possible to detect small changes in ribonucleic acids of large molecular weight. It had been considered necessary that the initial fractionation should involve the separation of oligonucleotides according to their total charge at pH 7.5 (and thus, according to their chain length). Tomlinson and Tener (1962, 1963) had already demonstrated that the use of 7 M urea in DEAE-cellulose chromatography minimized the secondary binding forces and permitted the fractionation of oligonucleotides essentially in accordance with their total charge. Subsequently, the use of DEAE-Sephadex in place of DEAE-cellulose in this technique was shown to reduce the nonionic binding even further, permitting the fractionation, according to chain length, of smaller oligonucleotides from ribonuclease T_1 digests (Rushizky *et al.*, 1964). However, from the present studies, it is clear that, although the fractionation of such digests by the DEAE-Sephadex 7 M urea procedure results in complete resolution of groups of oligonucleotides up to the pentanucleotide level, the nonionic forces become a serious problem in resolution of oligonucleotides of larger size. Thus, it appears that, for an oligonucleotide of chain length six or more, its base composition is also a factor in determining its position of elution. In fact, other experiments in this laboratory have shown that, with this type of chromatography, a nonanucleotide of low purine content can be displaced from the average nonanucleotide elution position by a distance as large as that which normally corresponds to a change of one less negative charge (*i.e.*, a change in chain length of one less nucleotide).

This lack of complete resolution of larger oligonucleotides in the primary separations, however, was not a serious problem in the present studies since, in order to detect differences in the oligonucleotides produced by ribonuclease hydrolysis, the only requirement was that the chromatographic patterns of peaks and valleys should be reproducible. In the case of the subfractionation of the larger size groups the use of DEAE-Sephadex chromatography at low pH in the absence of urea represents a new technique which exploits two factors both of which are a consequence of the base content of the components of each group: the variable charge on the components induced by the low pH and the various nonionic forms of binding occurring between the components and the chromatographic medium. This concept has been used previously in the fractionation of tri- and tetranucleotides on Dowex 1 columns at low pH (Solymosy *et al.*, 1965).

Ribonuclease T_1 is a base-specific endonuclease which cleaves ribonucleic acid only at guanosine 3′-phosphoryl bonds. Thus, the observation that, after ribonuclease T_1 digestion of *ts*3 RNA, there are two relatively long oligonucleotides missing compared with the digest of the wild-type RNA suggests that *ts*3 RNA contains at least two substitutions of guanine for some other base, since no extra oligonucleotides larger than those in peak

13 were found in the digest of *ts*3 RNA compared with that of the MS2 RNA. However, it is conceivable that these observations were due to deletion mutations, although this seems unlikely if past experiences with the phage T4 are applicable in this case. In T4, spontaneously arising deletions or additions shift the reading frame (Crick *et al.*, 1961), and nitrous acid induced deletions are relatively long (Tessman, 1962). With either of these events the effect should be lethal in the case of phage MS2, as there is no evidence that this organism contains a nonessential gene. The substitutions by guanine could have occurred in the one spontaneous or any of the three nitrous acid mutational steps. However, since the phage was exposed to extensive treatment with nitrous acid and in view of the theoretical considerations (Lavallé, 1960) and experimental evidence that nitrous acid can induce the change adenine → guanine in single-stranded DNA (Tessman *et al.*, 1964), this transition appears to be the most reasonable explanation for the observations on the peak 13 from *ts*3 RNA discussed above.

In conclusion, the present work shows that small differences in high molecular weight ribonucleic acids can be detected, providing the method of cleavage used locates the points of difference in one or more of the larger oligonucleotides. Thus, new methods for the cleavage of RNA will be required before all possible point mutations could be detected. For example, for the analysis of pyrimidine-to-pyrimidine changes it would be appropriate to use a cleavage method which is specific for cytidine 3′-phosphoryl bonds, and this can be achieved by the chemical modification of ribonucleic acid followed by pancreatic ribonuclease digestion (Lee *et al.*, 1965; Ho and Gilham, 1967). It seems likely that the methods described here can be improved and automated such that they can be applied to the routine analysis of sequences in large ribonucleic acids. One obvious improvement would involve the double-labeling technique where a single digest of a mixture of the two ribonucleic acids in question could be analyzed with a dual-channel scintillation counter. Finally, the use of low-pH subfractionation as a preparative technique should permit the isolation and sequence analysis of larger oligonucleotides from digests of ribonucleic acids, and the determination of nucleotide sequences in the immediate vicinity of point mutations should also be possible.

References

Crick, F. H. C., Barnett, L., Brenner, S., and Watts-Tobin, R. J. (1961), *Nature 192*, 1227.

Davis, J. E., and Sinsheimer, R. L. (1963), *J. Mol. Biol. 6*, 203.

Enger, M. D., Stubbs, E. A., Mitra, S., and Kaesberg, P. (1963), *Proc. Natl. Acad. Sci. U. S. 49*, 857.

Fraenkel-Conrat, H., Singer, B., and Tsugita, A. (1961), *Virology 14*, 54.

Gilham, P. T., and Robinson, W. E. (1964), *J. Am. Chem. Soc. 86*, 4985.

Ho, N. W. Y., and Gilham, P. T. (1967), *Biochemistry 6*, 3632.

Katz, S., and Comb, D. G. (1963), *J. Biol. Chem. 238*, 3065.

Lavallé, R. (1960), *Compt. Rend. 250*, 1134.

Lee, J. C., Ho, N. W. Y., and Gilham, P. T. (1965), *Biochim. Biophys. Acta 95*, 503.

Robinson, W. E., and Gilham, P. T. (1967), *7th Intern. Congr. Biochem., Tokyo, Aug 19–25*, p 660.

Rushizky, G. W., Bartos, E. M., and Sober, H. A. (1964), *Biochemistry 3*, 626.

Rushizky, G. W., Greco, A. E., and Rogerson, D. L. (1965), *Biochim. Biophys. Acta 108*, 142.

Sinha, N. K., Fujimura, R. K., and Kaesberg, P. (1965), *J. Mol. Biol. 11*, 84.

Solymosy, F., Tener, G. M., and Reichmann, M. E. (1965), *Virology 27*, 409.

Strauss, J. H., and Sinsheimer, R. L. (1963), *J. Mol. Biol. 7*, 43.

Tessman, I. (1959), *Virology 9*, 375.

Tessman, I. (1962), *J. Mol. Biol. 5*, 442.

Tessman, I., Poddar, R. K., and Kumar, S. (1964), *J. Mol. Biol. 9*, 352.

Tomlinson, R. V., and Tener, G. M. (1962), *J. Am. Chem. Soc. 84*, 2644.

Tomlinson, R. V., and Tener, G. M. (1963), *Biochemistry 2*, 697.