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Determination of Viral Plus and Minus Ribonucleic Acid Strands by an Isotope Dilution Assay*

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ABSTRACT: The determination of radioactive viral plus and minus strands in the presence of labeled host cell ribonucleic acid (RNA) by an isotope dilution method is described. With use of this procedure it is shown that the ratio of viral plus to minus strands is about 8 in cells infected with MS2 phage and 1.5 in cells infected with MU9, an amber mutant of MS2. The method has been used elsewhere to demonstrate that the virus-specific RNA polymerase, Q_β replicase, when primed with Q_β plus strands first synthesizes minus strands and later predominantly plus strands (Weissmann, C., and Feix, G. (1966), *Proc. Nat. Acad. Sci. U. S. 55*, 1264), but when primed with Q_β minus strands, produces plus strands from the very outset of the reaction (Weissmann, C., Feix, G., Slor, H., and Pollet, R. (1967), *Proc. Nat. Acad. Sci. U. S. 57*, 1870).

The study of viral RNA synthesis in bacteria infected with RNA phages is complicated by the concomitant synthesis of host cell RNA. Whereas the synthesis of host RNA can be specifically reduced by ultraviolet irradiation (Fenwick *et al.*, 1964) or, if *Escherichia coli* spheroplasts are used, by treatment with actinomycin (Haywood and Sinsheimer, 1963), these procedures give rise to quantitative and even qualitative alterations in viral RNA synthesis (Fenwick *et al.*, 1964; Kelly *et al.*, 1965; Nonoyama and Ikeda, 1964; Haywood and Harris, 1966). Viral RNA synthesis has been studied by infectivity assays (Paranchych, 1963; Engelhardt and Zinder, 1964; Pfeifer *et al.*, 1964; Delius and Hofschneider, 1964) or by measuring the amount of radioactivity recovered in phage particles after labeling the infected host cells for a limited time with RNA precursors (Cooper and Zinder, 1963; Lodish *et al.*, 1965); however, the amount of information obtained by these procedures is limited, since it is not possible, for example, to compare the rate of synthesis of viral plus strands[1] with that of viral minus strands, double-stranded RNA, and host RNA.

The double isotope specific dilution assay described in this paper allows the simultaneous determination of radioactive viral plus and minus strands in the presence of labeled host RNA. In principle, the method is a further elaboration of the specific dilution assay used to identify both DNA (Hoyer *et al.*, 1964) and RNA (Yankofski and Spiegelman, 1963; Weissmann *et al.*, 1964b; Robinson *et al.*, 1964) species or to determine the distribution of radioactivity between the plus and minus strands of virus-specific double-stranded RNA (Weissmann, 1966; Billeter *et al.*, 1966b).

Results

In the following section the specific dilution assay, as applied to the analysis of labeled, double-stranded viral RNA, will be discussed in more detail than has been the case hitherto, because it is the basis for the more elaborate double isotope specific dilution assay.

Specific Dilution Assay. When double-stranded MS2

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[1] The term "plus" strand is used to denote a viral RNA strand of the parental type; "minus" strand, to denote the strand with a base sequence complementary to that of the plus strands; SSC, 0.15 M sodium chloride–0.015 M sodium citrate; $n\times$ SSC, n-fold concentrated SSC; A_{260} unit, quantity of material resulting in an absorbance of 1.0 when dissolved in 1.0 ml and read ta wavelength 260 mμ (1.0-cm light path).

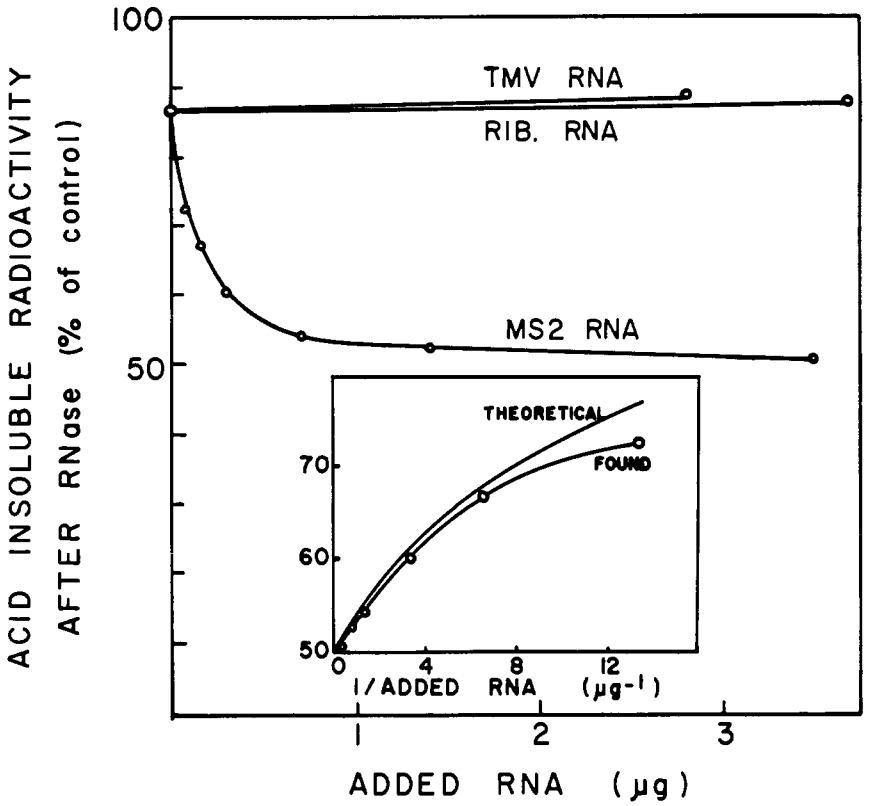

FIGURE 1: Specific dilution analysis of uniformly labeled double-stranded MS2 RNA. ^{32}P-labeled double-stranded RNA (0.17 μg, 2080 cpm) was heated and reannealed without further RNA additions or with varying additions of MS2 or TMV RNA, and the RNase-resistant radioactivity of each sample was determined as described in the Methods section. The values are expressed as per cent of the RNase-resistant radioactivity of an unheated sample. Inset: The same data, in a semireciprocal plot, allow extrapolation of radioactivity in minus strands, as set forth in Results.

RNA is heat denatured and reannealed in the presence of a small quantity of labeled MS2 RNA, a substantial fraction of the latter is converted into a double-stranded form. The specific dilution assay is based on the finding that this conversion can be almost completely suppressed by the addition of an excess of unlabeled MS2 RNA, but not of other RNAs such as TMV, *E. coli* ribosomal, or Q_β RNA. Thus, MS2 RNA but none of the other RNAs can compete with the labeled RNA for the limited amount of minus strands available for annealing, or, in other words, only MS2 RNA can dilute the radioactive MS2 RNA and reduce the amount of radioactivity incorporated into the double-stranded RNA. It appears that the dilution phenomenon occurs only if the labeled and the competing RNA species have extended base sequences in common, and the method may in fact be used to assess the extent of base sequence homology between different RNA species (Weissmann, 1966; Weissmann and Ochoa, 1967). The specific dilution effect can also be utilized to determine the distribution of radioactivity between the plus and minus strand of purified labeled double-stranded viral RNA; if the preparation is denatured and reannealed in the presence of a large excess of the homologous unlabeled plus strands, all radioactive plus strands are displaced from the duplex and only the radioactivity due to minus strands is recovered in a double-stranded form. Since double-stranded RNA is resistant to the action of ribonuclease under conditions where single-stranded RNA is sensitive (Geidushek *et al.*, 1962; Billeter *et al.*, 1966a), the displacement (dilution) of radioactive strands from the duplex can be followed by determining the RNase-resistant radioactivity before and after the heating procedure. It should be noted in this connection that reannealing of minus strands goes virtually to completion when carried out in the presence of an excess of plus strands, while the reannealing of double-stranded RNA by itself is only about 85–90% complete under optimal conditions (Billeter *et al.*, 1966a). In order to determine the distribution of radioactivity between the two strands of virus-specific, double-stranded RNA, the dilution assay is carried out as follows. A series of identical samples is denatured and reannealed in the presence of increasing amounts of nonlabeled plus strands, so that radioactive plus strands are diluted out while radioactive minus strands reanneal completely. Radioactivity in minus strands is then extrapolated as the radioactivity remaining RNase resistant after annealing in the presence of an "infinite" amount

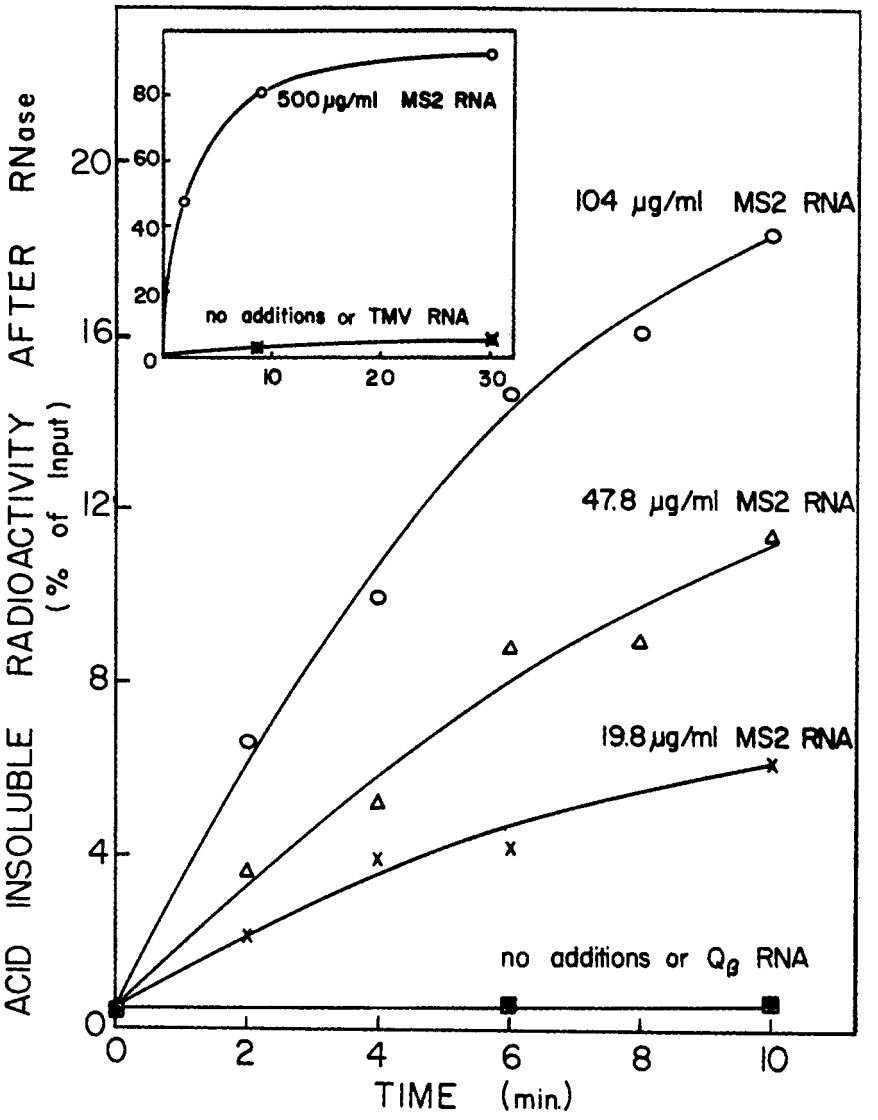

FIGURE 2: Demonstration that radioactive double-stranded RNA from MS2-infected cells, after heat denaturation and reannealing in the presence of excess MS2 RNA, contains radioactivity exclusively in MS2 minus strands (for explanation, see Results section). ^{3}H-labeled double-stranded RNA from MS2-infected cells was heat denatured and reannealed in the presence of a large excess of unlabeled MS2 RNA. The product was treated with RNase and the RNase-resistant RNA was reisolated. It was denatured by heating in water for 7 min at 100° at a final concentration of 0.7 μg/ml. Initial rates of reannealing were determined as follows. Aliquots (0.13 μg, 1800 cpm) were heated at 72° in 0.2 ml of 0.2× SSC with the additions indicated in the figure. After varying periods of time, the samples were chilled, the salt concentration was brought to SSC, and the RNA was digested with 50 μg/ml of RNase A and 25 units/ml of T_1 RNase in a final volume of 2 ml, for 30 min at 25°. Acid-insoluble radioactivity was determined as described in the Methods section and expressed as per cent of total input radioactivity. Inset: The annealing experiment was carried out as above; however, MS2 RNA was added at a high concentration, in order to determine the maximum extent of the annealing reaction.

of nonlabeled plus strands. The extrapolation is described in the Methods section.

Figure 1 shows, as an example, the analysis of uniformly labeled, double-stranded MS2 RNA by the specific dilution assay. It may be noted that after heating and reannealing double-stranded RNA without any added viral RNA, only 85% of the original RNase-resistant radioactivity was recovered, due to incomplete reannealing, as mentioned above. This causes a deviation from the theoretical dilution curve (inset to Figure 1) for low amounts of added RNA. With increasing amounts of added RNA, the fit becomes satisfactory and the extrapolated value for radioactivity in minus strands is 49%, in close agreement with the expected value of 50%.

The question might be raised as to whether the

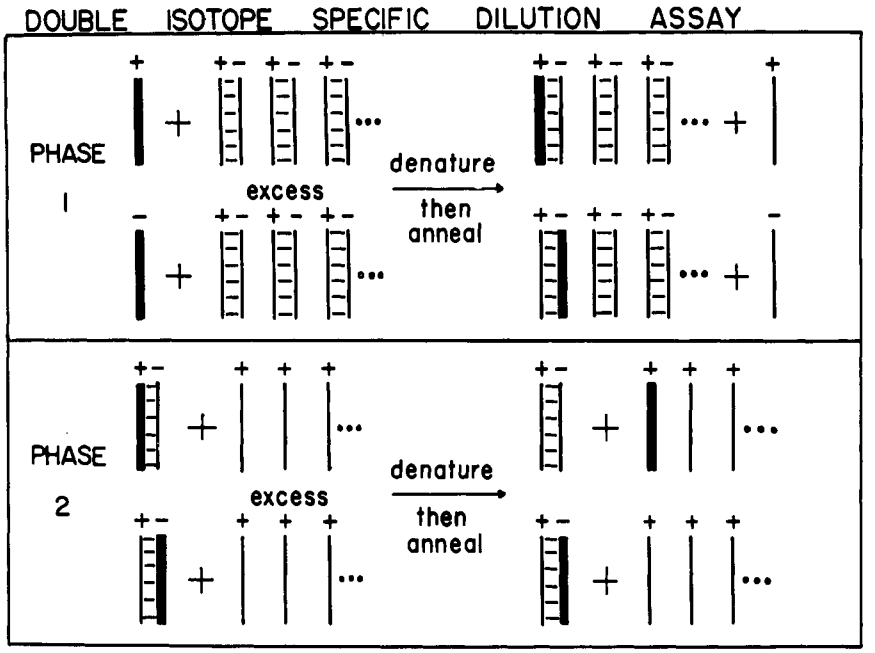

FIGURE 3: Determination of radioactive viral plus and minus strands by an isotope dilution assay. In principle, the assay may be carried out in two phases (Weissmann, 1965). Phase 1: Radioactive viral plus and minus strands are annealed with an excess of denatured, homologous double-stranded RNA and are thereby rendered RNase resistant, while any nonviral RNA remains RNase sensitive. Phase 2: The double-stranded product is denatured and reannealed in the presence of an excess of unlabeled plus strands. The radioactive plus strands are thereby diluted out of the duplex, becoming RNase sensitive, while the minus strands remain RNase resistant. In the double isotope specific dilution assay both steps are combined, and some viral RNA, labeled with a different isotope, is added to the sample as an internal standard, thereby permitting the quantification of the results.

radioactive RNA remaining RNase-resistant under these assay conditions indeed consists of viral minus strands. It could be argued that (1) not all double-stranded RNA is completely denatured, and that therefore some of the RNase-resistant radioactive RNA is still due to plus strands, that (2) radioactive plus strands may in some fashion interact with other plus strands and give rise to RNase-resistant RNA, or that (3) the preparation is contaminated with a radioactive double-stranded RNA, not related to the viral RNA under investigation, which after denaturation reanneals to re-form an RNase-resistant RNA. The first possibility has been excluded earlier by the demonstration that under the conditions of the specific dilution assay complete mixing of the plus strands of double-stranded and added labeled plus strands is attained (Billeter *et al.*, 1966a). The second objection was met by showing, both for MS2 and Q_β RNA, that there is no interaction between plus strands (intact or fragmented) leading to the formation of RNase-resistant RNA (Weissmann *et al.*, 1964a; Weissmann, 1967) under the conditions of the specific dilution assay. In order to eliminate possibility 3, advantage is taken of the fact that the rate of reannealing of denatured double-stranded RNA is concentration dependent, as is that of DNA (Inman and Baldwin, 1964). Since the rate-limiting step in denaturation is presumably the collision of complementary strands, the rate of annealing should depend on the concentration of both plus and minus strands. Therefore, if one denatures double-stranded RNA labeled exclusively in the minus strand and determines the rate of reannealing of the radioactive RNA at different concentrations of unlabeled plus strands, one should find it proportional to the plus strand concentration. Conversely, if the rate of annealing of a labeled RNA is found to be specifically accelerated by the addition of a certain RNA, this may be taken as evidence that the two have a complementary base sequence. In the experiment of Figure 2 labeled double-stranded RNA obtained from MS2-infected cells was heated and reannealed in the presence of a large excess of cold MS2 RNA (*i.e.*, subjected to a procedure similar to the specific dilution assay); the product was treated with RNase and reisolated by Sephadex chromatography. This double-stranded RNA (putatively labeled only in the minus MS2 strand) was denatured and aliquots were heated, at low concentrations, with varying amounts of added, unlabeled MS2 RNA for different lengths of time. In the absence of added MS2 RNA, no reannealing was detectable. In the presence of 20, 50, or 100 μg/ml of MS2 RNA, the initial rates of annealing were about proportional to the plus strand concentration (Figure 2). In the presence

of 500 μg/ml of MS2 RNA, over 90% of the minus strands were annealed after 30 min (Figure 2, inset). Q_β RNA or TMV RNA did not affect the rate of reannealing. If any double-stranded RNA other than that of MS2 had been present, it would have been expected (depending on its concentration and its rate constant of reannealing) to either (a) anneal in the absence of MS2 RNA, or (b) not anneal, whether or not MS2 RNA was added. Since in fact no annealing was observed in the absence of MS2 RNA, and virtually complete annealing occurred in its presence, one may conclude that no non-MS2 RNA was present, and, in view of the arguments presented above, it follows that virtually all of the radioactivity was due to MS2 minus strands, and that the interpretation of the specific dilution assay given above is justified.

The Double Isotope Specific Dilution Assay. This assay is based on the techniques described above and permits the determination of radioactive plus and minus strands in the presence of radioactive, nonviral RNA. The principle of the method is shown in Figure 3. Radioactive viral RNA (both plus and minus strands) is converted into a double-stranded form by annealing with an excess of denatured, unlabeled double-stranded viral RNA; under appropriate conditions, 85–90% of the labeled viral RNA becomes RNase resistant. The labeled, double-stranded RNA thus obtained is then analyzed by the specific dilution assay to determine the distribution of radioactivity between plus and minus strands. In the double-isotope assay authentic viral RNA, labeled with an isotope different from the one used for the unknown sample, is added as an internal standard in order to permit quantification of the results. In practice, the steps just outlined are combined. The unknown RNA sample, usually ^{14}C labeled, is mixed with ^{32}P-labeled viral RNA and an amount of double-stranded viral RNA which is in about tenfold or greater excess over the viral RNA estimated to be already present. Aliquots of the mixture, both without further additions and with addition of varying amounts of unlabeled viral RNA (ranging from about $^1/_{10}$ to 20 times the amount of the double-stranded RNA), are heated above the T_m of the double-stranded RNA and then subjected to annealing conditions. The RNase-resistant ^{32}P and ^{14}C radioactivities are determined and, after appropriate correction for blank values, expressed as fractions (f_p and f_c, respectively) of the input radioactivities. A plot of f_c against the corresponding f_p values gives a straight line, with the slope and the intercept indicating the fraction of ^{14}C radioactivity in plus strands and minus strands, respectively (see Methods section). The method has been tested by reconstruction experiments in which ^{14}C-labeled plus strands, minus strands, and *E. coli* RNA were mixed in different proportions and then analyzed. Table I shows the results of a set of ten experiments. It is seen that within a wide range of combinations, the values found for plus and minus strands reflect the input ratios with reasonable accuracy; the values for minus strands show a mean relative actual error of −2.9% ($\sigma_{\bar{x}}$ = 1.5%), those for "plus" strands one of −5.5% ($\sigma_{\bar{x}}$ = 2.3%). Two possible sources of error have been noted. (1) When a sample contains high concentrations of labeled *E. coli* RNA, the heating and annealing procedure may give rise to abnormally high values (5–6%) of apparently RNase-resistant radioactivity. However, the routine use of RNase T_1, along with pancreatic RNase A, in the RNase digestion step reduces the values to a normal level (<0.5%). When it is necessary to measure low levels of minus strands (1% of the total radioactivity or less) it is advisable to use, as a control, RNA from uninfected cells labeled and processed under the same conditions as the sample to be analyzed, in order to determine the blank values for minus and plus strands. (2) The dilution assay is based on the relative extent to which ^{14}C- and ^{32}P-labeled plus strands are prevented from becoming double stranded by excess unlabeled plus strands. If one of the labeled RNAs were partially degraded, it might anneal less well, and therefore be excluded more readily from the duplex than the other, intact

TABLE I: Double Isotope Specific Dilution Assay on Mixtures of Known Composition of Viral Plus and Minus Strands and *E. coli* RNA.[a]

	Minus Strand[b]		Plus Strand[b]	
Expt	Input	Found	Input	Found
1	0	0.6	0	−0.6
2	10.6	10.2	10.6	9.7
3	14.4	15.0	14.4	14.4
4	22.6	21.2	22.6	20.6
5	28.8	29.2	28.8	23.1
6	30.6	31.1	30.6	29.1
7	50.0	49.1	50.0	46.9
8	7.5	6.8	82.6	86.2
9	11.8	10.9	70.3	70.8
10	17.6	16.8	61.4	57.5

[a] Each sample contained about 1000 cpm of [^{14}C]-RNA. Samples were prepared by mixing ^{14}C-labeled MS2 RNA, ^{14}C-labeled double-stranded RNA, and ^{14}C-labeled *E. coli* RNA to give the desired composition. The assay and the calculations were carried out as described in the text. The mean relative actual error

$$E = \frac{\sum \frac{\text{input} - \text{found}}{\text{input}}}{N}$$

was −2.9% for minus strands and −5.5% for plus strands; the standard errors of the mean ($\sigma_{\bar{x}}$) were 1.5 and 2.3%, respectively. [b] Values in per cent.

$$\sigma_{\bar{x}} = \frac{\sigma}{\sqrt{N-1}}, \text{ where } \sigma = \sqrt{\frac{\sum\left[E - \frac{(\text{input-found})}{\text{input}}\right]^2}{N}}$$

RNA, thereby distorting the results of the assay. This possibility was tested by preparing ^{32}P-labeled MS2 RNA of high molecular weight (27 S) and ^{14}C-labeled MS2 RNA of low molecular weight (about 4–6 S) and subjecting a mixture of the two to the double isotope specific dilution assay. As shown in Figure 4, the partially degraded RNA is displaced more readily from the duplex, so that the plot of ^{14}C radioactivity *vs.* ^{32}P radioactivity yields a concave rather than a straight line. An approximate value for plus strands can nonetheless be obtained in these cases by extrapolating the intercept and determining the slope of a straight line drawn through the point of interception and the point on the curve corresponding to the sample which has been heated and reannealed without added viral RNA. This procedure is indicated in Figure 4.[2] We have not encountered such a situation in practice; however, it is necessary to prepare and store all RNA samples with appropriate care (see Methods section).

Two examples demonstrate the application of the method. Figure 5A shows the results of the double isotope specific dilution assay carried out on the RNA of MS2 infected cells, labeled with [^{14}C]uracil from infection until 40 min after infection. It is seen that virus-specific RNA comprises 32% of the total labeled RNA, and that the ratio of plus to minus strands is about 8. In the case of *E. coli* infected with MU9, an amber mutant of MS2, 43% of the labeled RNA is virus specific, and the ratio of plus to minus strands is 1.5 (Figure 5B). There is thus a remarkable overproduction of minus strands. The mutant is similar to the mutant sus-11 of Lodish *et al.* (1964) in that it synthesizes excessive amounts of virus-induced RNA polymerase and of virus-specific double-stranded RNA (Lodish and Zinder, 1966).

Discussion and Summary

An isotope dilution method has been developed which allows the determination of radioactive viral plus and minus strands in the presence of labeled host cell RNA. The validity of the analysis was demonstrated on mixtures containing radioactive plus and minus strands as well as *E. coli* RNA, in known proportions. Using the double isotope specific dilution assay, it was shown that during the first 40 min after infection of *E. coli* Hfr 3000 with phage MS2, 32% of the newly synthesized RNA is virus specific and that the ratio of plus to minus strands is about 8. After infection with MU9, an amber mutant of MS2, abnormally large quantities of minus strands are produced in the nonpermissive host. The method has been extensively used to analyze the product of *in vitro* RNA synthesis by viral RNA polymerase (replicase, synthetase) and was instrumental in demonstrating that Q_β replicase primed by Q_β plus strands first synthesizes viral minus strands and later predominantly Q_β plus strands, whereas, when primed by single-stranded Q_β minus strands, the enzyme gives rise to plus strands from the very beginning of the incubation (Weissmann *et al.*, 1967; Feix *et al.*, 1967b). A simpler form of the assay (specific dilution assay) has been used to show asymmetric labeling of double-stranded RNA extracted from MS2-infected *E. coli* which had been subjected to a short pulse of radioactive guanine (Billeter *et al.*, 1966b). Appropriate modifications of the double isotope specific dilution assay allowed the demonstration that there is no base sequence homology between the RNAs of the phages MS2 and Q_β, while there is extensive overlap between MS2 and f_2 RNA (Weissmann and Ochoa, 1967; Feix *et al.*, 1967a).

Experimental Procedures

Materials and Preparations. Materials were purchased from the sources indicated previously (Billeter and Weissmann, 1966; Billeter *et al.*, 1966a). Annealing tubes were prepared by sealing off EDTA-washed glass tubing (outer diameter, 8 mm; wall thickness, 1 mm; and length, 10 cm) to give a conical bottom.

Bacteriophage MS2 and its host, *E. coli* Hfr 3000, were obtained from Dr. A. J. Clark, University of California, and bacteriophage f_2 from Dr. N. Zinder, The Rockefeller University. Phage Q_β was provided by Professor I. Watanabe, Tokyo. The viruses were propagated as previously described (Billeter and Weissmann, 1966). The amber mutant MU9 was obtained by nitrous acid treatment of MS2 by Dr. C. I. Davern, Cold Spring Harbor Biological Laboratory, and was propagated on the permissive host *E. coli* K90. Great care was taken to eliminate traces of RNase from glassware and solutions. Glassware and, whenever possible, solutions were autoclaved (30 min at 20 psi), and cleaned glassware was handled with gloves only, to avoid contamination. Both MS2 and Q_β were purified by a procedure similar to that described for phage MS2 by Strauss and Sinsheimer (1963). Q_β differs from MS2 in that it forms a precipitate band during the CsCl density gradient centrifugation step, but it redissolves on dilution with water. Viral RNA was extracted by the method of Gesteland and Boedtker (1964), however, using 1% SDS, and Macaloid (one-tenth the weight of the virus) rather than bentonite. It was stored as a precipitate in 66% ethanol at −70° and dissolved in 0.02 M Tris-HCl buffer (pH 7.2) prior to use.

Radioactive viral RNA was prepared as described previously (Weissmann *et al.*, 1964a). Viral RNA fragments were isolated by sucrose gradient centrifuga-

[2] This approximative procedure is justified by the following consideration. The most meaningful values obtained in the assay are (a) the one corresponding to the addition of the largest quantity of unlabeled viral RNA, because under these conditions almost exclusively labeled minus strands are being measured, and competition between plus strands of differing size are then irrelevant, and (b) the one obtained when no unlabeled viral RNA is added, because then the shorter plus strands experience the smallest degree of competition by longer plus strands. If a straight line is drawn through the point of interception, which can be extrapolated from a, and through point b, it is as though a double isotope specific dilution assay with only two points had been performed, and intercept and slope of this line are interpreted as usual.

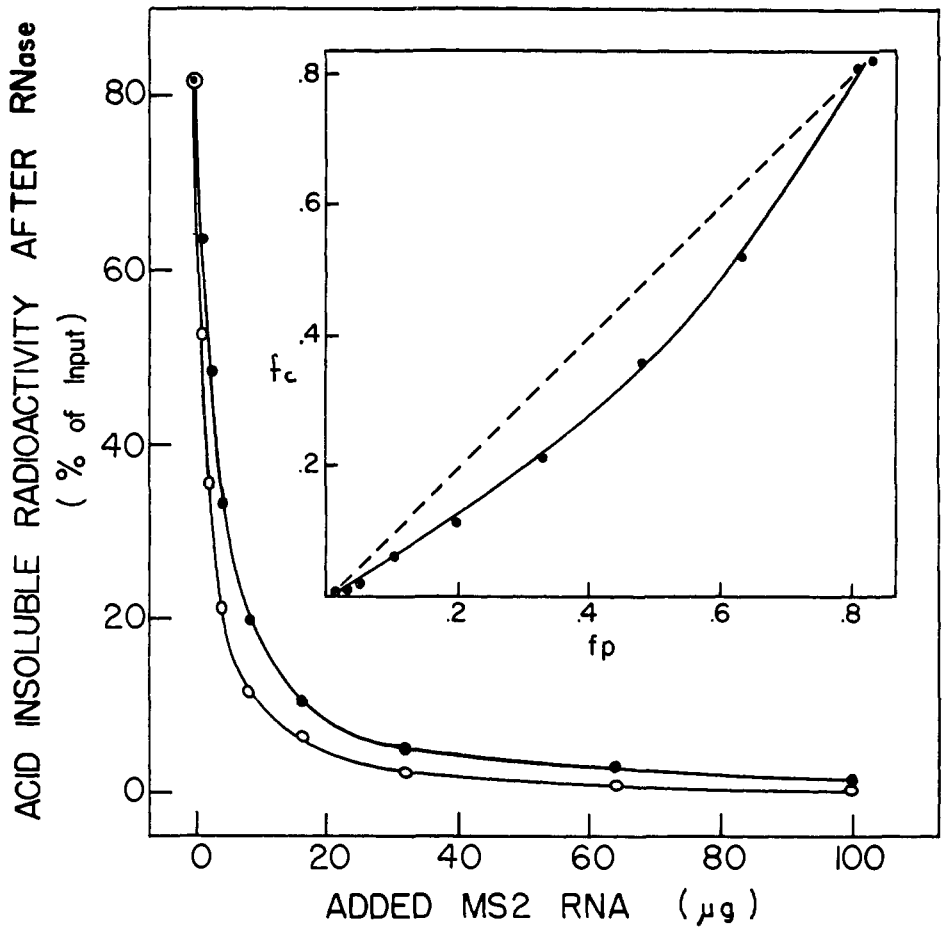

FIGURE 4: Influence of the molecular weight of the viral RNA on its annealing capacity. Samples containing 27S ^{32}P-labeled MS2 RNA (0.1 μg, 1000 cpm), 4–6S ^{14}C-labeled MS2 RNA (0.5 μg, 2000 cpm), and 10 μg of unlabeled double-stranded RNA were heated and annealed with the amount of unlabeled MS2 RNA (27 S) indicated. RNase-resistant ^{14}C and ^{32}P radioactivities (after subtraction of the blank values) are expressed as fraction of the input radioactivities and plotted against the amount of added, unlabeled MS2 RNA. (○—○) ^{14}C radioactivity; (●—●) ^{32}P radioactivity. Inset: The ^{14}C values are plotted against the corresponding ^{32}P values. A concave line (●—●) results, indicating that the partially degraded [^{14}C]RNA is displaced more readily than the intact [^{32}P]RNA. Had the two RNAs been equivalent, a straight line (- - - - -) would have resulted.

tion from partially degraded RNA preparations. Double-stranded MS2 and Q_β RNA, both unlabeled and uniformly labeled, were prepared as previously described (Billeter *et al.*, 1966a).

Radioactive nucleic acids from infected or non-infected *E. coli* were prepared according to Billeter *et al.* (1966b). The preparations were freed of DNA by incubation for 15 min at 25° with 20 μg/ml of electrophoretically purified pancreatic DNase (Worthington), in 0.05 M Tris-HCl buffer (pH 7.3) containing 0.005 M $MgCl_2$, at a concentration of 20 A_{260} units/ml or less. Following phenol extraction and alcohol precipitation, the RNA was chromatographed on Sephadex G-200 with standard saline citrate (SSC) or 0.5 M ammonium bicarbonate (pH 8.4) as eluent, in order to remove acid-precipitable DNA fragments.

RNA concentrations were determined spectrophotometrically assuming an $\epsilon_{260}^{1\%}$ of 251 dl g^{-1} cm^{-1} for MS2 RNA (Strauss and Sinsheimer, 1963) and of 210 dl g^{-1} cm^{-1} for double-stranded MS2 RNA (Billeter *et al.*, 1966b).

Double-stranded MS2 RNA, which, as shown in the preceding section, is labeled exclusively in the minus strand, was prepared as follows. Double-stranded MS2 RNA uniformly labeled with [^{3}H]uracil (100 μg, 3.1 × 10^6 cpm) was diluted to 120 ml (final concentration, 0.08× SSC) and heated for 4 min at 95°. This was followed by the addition of unlabeled MS2 RNA (3.8 mg) and 0.15 ml of 10× SSC. The solution was lyophilized to dryness and the residue was taken up in 1.1 ml of water and annealed for 1 hr at 75°. After dilution of the preparation to 10 ml the salt concentration was adjusted to 1× SSC and 50 μg of pancreatic RNase A/ml was added. The solution was incubated for 30 min at 25° and then extracted four times with two volumes of phenol. The phenol

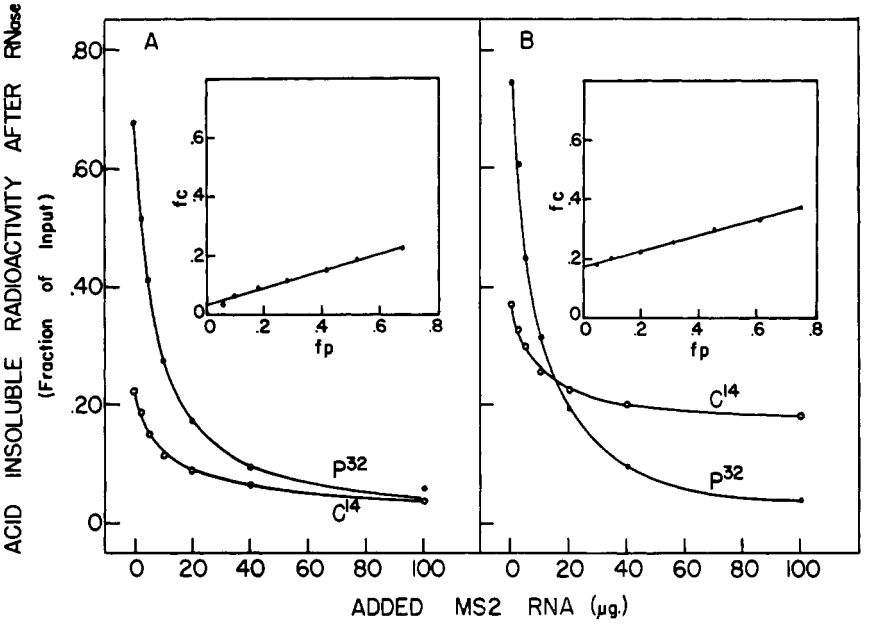

FIGURE 5: Double isotope specific dilution assay. Analysis of ^{14}C-labeled RNA from *E. coli*, infected with MS2 (A) or the mutant MU9 (B). Hfr 3000 infected with either MS2 or MU9 was uniformly labeled with [^{14}C]uracil from infection until 40 min later (Billeter and Weissmann, 1966). The RNA was purified as outlined in Methods. ^{14}C-labeled sample (1000 cpm), 1000 cpm of ^{32}P-labeled MS2 RNA, 10 μg of double-stranded MS2 RNA, and unlabeled MS2 RNA as indicated were heated, annealed, and processed as described in the Methods section. RNase-resistant radioactivities (after correction for the blank or core values) are expressed as fraction of the input radioactivities and plotted against the amount of added unlabeled MS2 RNA. Insets: The ^{14}C values are plotted against the corresponding ^{32}P-values. Slope and intercept give the fraction of ^{14}C radioactivity in plus and minus strands, respectively (slope in A, 0.28, and in B, 0.26; intercept in A, 0.035, and in B, 0.18).

was removed with ether, and the nucleic acid was precipitated by the addition of two volumes of ethanol, dissolved in 5 ml of SSC, and chromatographed on a column of Sephadex G-200 (1.8 × 60 cm). The front-running fractions contained the double-stranded RNA (about 80 μg and 1.2 × 10^6 cpm). When subjected to the specific dilution test, no radioactivity was displaced by a 50-fold excess of cold MS2 RNA.

The Specific Dilution Assay. Determination of the Distribution of Radioactivity between the Plus and Minus Strand of Virus-Specific Double-Stranded RNA. Identical aliquots of purified ^{32}P-labeled double-stranded MS2 RNA (2100 cpm, 0.17 μg) were distributed into annealing tubes. Tubes 1–4 had no further additions; tubes 5–10 received 0.075, 0.15, 0.3, 0.7, 1.4, and 3.5 μg of MS2 RNA, respectively. Sufficient SSC was added so that each tube contained 7.5 μmoles of NaCl and 0.75 μmole of sodium citrate. The samples were then evaporated to dryness in a desiccator at 12 mm over P_2O_5. (If ammonium bicarbonate was present, it was subsequently removed by exposing the samples to high vacuum (0.5 mm) for 6–12 hr.) The residue was dissolved in 20 μl of water and the tubes were sealed. Samples 3–10 were kept for 1 min at 85°, 3 min at 120°, and 1 hr at 85°. The contents of all tubes were quantitatively transferred to test tubes using 2 ml of SSC. All samples but 1 and 3 were digested with 50 μg/ml of pancreatic RNase A and 25 units/ml (Takahashi, 1961) of RNase T_1 (Sankyo, Tokyo) for 30 min at 25°. Albumin (100 μg) and trichloroacetic acid (final concentration, 6%) were added to the chilled samples, and the resulting precipitates were collected and washed on Millipore filters with chilled, 6% trichloroacetic acid. The samples were dried overnight at 60° and the radioactivities were determined using a liquid scintillation spectrometer, as described elsewhere (Weissmann, 1965). In order to obtain a blank ("core"), a further aliquot of the double-stranded RNA was diluted to 2 ml with 0.005 M EDTA, heated for 5 min in a boiling water bath, and then rapidly chilled in an ice bath. After addition of NaCl and sodium citrate to a final concentration corresponding to SSC, nuclease digestion and plating were carried out as above. After subtraction of the blank (less than 1% of the input radioactivity from *all* values (this procedure is justified in footnote 3), the RNase-resistant radioactivities were expressed as fractions of the input radioactivity (sample 3). A comparison of samples 1 and 3 showed that the heating procedure alone did not render any radioactivity acid soluble. All determinations were carried out in duplicate.

The quantitative evaluation of the specific dilution assay is based on the following considerations. Let M = amount of minus strands, P = amount of plus

strands added to sample, M^* = radioactivity in minus strands, P^* = radioactivity in plus strands, R^* = acid-insoluble radioactivity after standard RNase digestion, T^* = total input radioactivity, and ϵ = recovery factor = amount of double-stranded RNA detected by RNase assay/theoretical amount that can be formed.

The total amount of plus strands in a tube is due both to the labeled plus strands present in the double-stranded RNA (which is equivalent to the amount of minus strands) and the unlabeled plus strands added. The specific radioactivity of plus strands will therefore be $P^*/(P + M)$. In the presence of an excess of plus strands, all minus strands could be annealed, as far as the stoichiometry of the reaction is concerned. If formation and detection of double-stranded RNA occurs with an efficiency ϵ, the contribution of radioactive minus strands to RNase-resistant radioactivity will be ϵM^*. Furthermore, as many plus strands as there are minus strands can be annealed. Taking into account the specific radioactivity of the plus strands and the recovery factor, the amount of RNase-resistant radioactivity due to plus strands is $\epsilon P^*M/(P + M)$. The total RNase-resistant radioactivity is therefore

$$R^* = \epsilon\frac{P^*M}{P + M} + \epsilon M^* \qquad (1)$$

When an excess of unlabeled plus strands is added

$$P > M, \text{ and } R^* \simeq \epsilon\frac{P^*M}{P} + \epsilon M^* \qquad (2)$$

so that a plot of R^* *vs.* $1/P$ is approximately linear and intercepts the ordinate at a value corresponding to ϵM^*.

The annealing of minus strands in the presence of an excess of plus strands is virtually complete (Billeter *et al.*, 1966a). However, under the conditions of the standard RNase assay, about 6–8% of the double-stranded RNA is degraded; ϵ is therefore about 0.92–0.94 (depending somewhat on the batch of RNase used). From the acid-insoluble radioactivity of a sample of pure, labeled double-stranded RNA measured before (T^*) and after the RNase treatment (ϵT^*), ϵ can be determined. (It is convenient to express the RNase-resistant radioactivities determined in the dilution test as the fraction of input radioactivity which has become RNase resistant. Equation 1 then becomes

$$\frac{R^*}{\epsilon T^*} = \frac{P^*M}{(P + M)T^*} + \frac{M^*}{T^*} \qquad (3)$$

and the intercept is read out as fraction of input radioactivity due to radioactive minus strands.)

The Double Isotope Specific Dilution Test. The following section describes a typical analysis of ^{14}C-labeled RNA of *E. coli* Hfr 3000 infected with MS2, prepared as outlined above. A solution was prepared, containing in 2 ml (final concentration, 0.5× SSC) about 40,000 cpm of the radioactive sample (40 A_{260} units or less), 20,000 cpm of ^{32}P-labeled MS2 RNA (10 μg or less), and 200 μg of double-stranded MS2 RNA. Samples (0.1 ml) of the mixture were distributed into 16 annealing tubes. Tubes 1–6 had no further additions; tubes 7–14 received 1, 2, 4, 8, 16, 32, 64, and 100 μg of MS2 RNA, respectively. All samples were evaporated to dryness as described above, and the residues were dissolved, each in 20 μl of water. Samples 3–16 were heated to 120 and 85° as described for the specific dilution assay. The total acid-insoluble radioactivities (T_p^* and T_c^*) of samples 1–4 were determined; there was no loss of acid-isoluble radioactivity following the heating procedure. Samples 1–14 were transferred to test tubes with 2 ml of 1× SSC, samples 15 and 16 with 2 ml of water. Samples 15 and 16 were heat denatured for 5 min at 100°, adjusted to 1× SSC, and digested with RNase as described above in order to determine the RNase-resistant "core." The RNase-resistant ^{32}P and ^{14}C radioactivities of samples 5–14 were also determined as described for the specific dilution assay. The values thus obtained (R'^*_p and R'^*_c) were corrected for the "core" to give the corrected RNase resistance R^*_p and R^*_c[3] and expressed as fractions (f_p and f_c) of the corresponding, total input radioactivities T^*_p and T^*_c, $f_p = R^*_p/T^*_p$, and $f_c = R^*_c/T^*_c$.

The Evaluation of the Data. The following expressions are derived as was eq 1

$$R^*_c = \epsilon\frac{P^*_c}{\sum P}M + \epsilon M^*_c \qquad (4)$$

$$R^*_p = \epsilon\frac{P^*_p}{\sum P}M \qquad (5)$$

where ΣP denotes the total amount of plus strands (whatever their origin) present in the sample. There is no second term in eq 5, because there are no ^{32}P-labeled minus strands in the purified viral RNA (Weissmann *et al.*, 1964a; Weissmann, 1967). Noting

[3] Let R'^* = measured RNase-resistant radioactivity of sample, R^* = RNase-resistant radioactivity due to double-stranded RNA, T^* = total radioactivity of sample, and c^* = RNase-resistant radioactivity of heat-denatured sample. Since the measured RNase-resistant radioactivity R'^* is due to double-stranded RNA and to a nondigestible core derived from the RNase-sensitive part of the labeled sample, we may write

$$R'^* = R^* + \frac{(T^* - R^*)}{T^*}c^*$$

$$R^* = \frac{T^*(R'^* - c^*)}{T^* - c^*}$$

The values of f required for eq 7 may be obtained directly.

$$f = \frac{R^*}{T^*} = \frac{R'^* - c^*}{T^* - c^*}$$

that $R^*_p/P^*_p = f_p$, eq 4 and 5 may be combined to give

$$R^*_c = f_p P^*_c + \epsilon M^*_c \quad (6)$$

Dividing by T^*_c yields

$$f_c = f_p \frac{P^*_c}{T^*_c} + \epsilon \frac{M^*_c}{T^*_c} \quad (7)$$

If f_c, the fraction of ^{14}C radioactivity converted into an RNase-resistant form, is plotted as a function of f_p, the corresponding ^{32}P value, a straight line results, the slope of which corresponds to the fraction of ^{14}C radioactivity present in plus strands, while the intercept, divided by ϵ, gives the fraction of ^{14}C radioactivity in minus strands. As mentioned above, under the conditions of the assay, ϵ is about 0.92–0.94, and is due to the low but measurable susceptibility of double-stranded RNA to RNase digestion (Billeter *et al.*, 1966a).

The evaluation of the data, including determination of slope and intercept by the least-squares method, was carried out by computer. A program (in BASIC) is available on request.

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