type of the smooth colonies was identified immunologically using the Quellung method and rabbit antiserum.

Results and Discussion.—A total of five different experiments was performed. The results are presented in Table 2.

It is evident from Table 2 that, in 31% (26/83) of the mice which were injected with both donor and recipient cultures in the absence of exogenous DNase, at least one transformation event had taken place. It can also be concluded that genetic recombination was due to DNA-mediated transformation, since no recombination took place when DNase was added to the system.

These experiments demonstrate that DNA-mediated transformation can occur spontaneously in genetically mixed populations of pneumococci growing in a living host. A more detailed discussion of the significance of transformation as a natural method of recombination for some microbial species was presented previously. 7

In addition to studies on pneumococcus and streptococcus, spontaneously occurring DNA-mediated transformation in vitro has since been observed in B. subtilis 10 and in H. influenzae. 11 It is probable therefore that genetic transformation may play a more widespread role in microbial recombinations in nature than has been supposed.

Summary.—DNA-mediated transformation occurs spontaneously among genetically-marked pneumococci growing together in a living host, the mouse.

We wish to acknowledge the competent technical assistance of Miss Ethel Stolnitz.

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† Postdoctoral research fellow, American Cancer Society, grant PRS-8.
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9 Hotchkiss, R. D., these PROCEEDINGS, 40, 49 (1954).

STUDIES ON THE FORMATION OF TOBACCO MOSAIC VIRUS RIBONUCLEIC ACID, III. UTILIZATION OF RIBONUCLEOSIDES OF HOST RIBONUCLEIC ACID*

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The process by which TMV-RNA 1 replicates within the cell is not understood, either with regard to mechanism or site of formation. Even though its synthesis is not mediated by cell DNA, 2 it is still possible that the nucleus is the site of its
formation. Since the formation of considerable amounts of nucleic acid possessing the properties of TMV-RNA is not the normal function of the cell, the materials needed for its synthesis might not be present in adequate amounts in the host cell. Hence, the prerequisite for its formation is the availability of the adequate amounts of the building blocks, which could be provided either by de novo synthesis or by the degradation of the host RNA. The evidence in support of the latter was presented in the previous paper. Following infection with TMV, microsomal RNA of the cytoplasmic fraction, which forms about 55–60 per cent of the total tobacco leaf RNA, is rapidly degraded and its degradation products are utilized in the synthesis of TMV-RNA. The evidence presented in this paper further demonstrates that the ribonucleoside moieties of the microsomal RNA are utilized in the synthesis of TMV-RNA.

Experimental.—TMV inoculum for infecting tobacco plants: The common strain of TMV used in this investigation was kindly supplied by Dr. F. O. Holmes of the Rockefeller Institute. TMV inoculum used for infecting the tobacco plants was prepared as previously described.

Plants: Tobacco plants (Nicotiana tabacum var. Turkish) used in these studies were grown in a greenhouse. These were kindly provided by Dr. Armin Braun of the Rockefeller Institute.

Growth of infected tobacco plants in Hoagland’s medium containing Pi32: The tobacco plants, 4–5 wks old, were transferred from the greenhouse to a room in which the temperature was maintained at 26°. Plants of same size and general appearance were selected, and bottom leaves were removed and discarded. The remaining leaves were dusted with carborundum and were rubbed with TMV inoculum. The leaves were afterward rinsed with water. The plants were immediately removed from the pots, and their roots were washed with tap water. One plant was placed in each bottle containing 80 ml of Hoagland’s nutrient solution to which was added Pi32 (amounts of Pi32 per ml of the nutrient solution are given in Tables 1 and 2). The phosphate content of the Hoagland’s nutrient solution was reduced to one tenth of that prescribed to ensure the maximum uptake of Pi32 by the plants. The nutrient solution was fortied by the weekly addition of 0.1 ml of 0.5% ferric tartrate to each bottle. The nutrient solution was gently aerated throughout the experimental period. The level of the solution in the bottles was maintained by the frequent addition of the nutrient solution containing Pi32. The bottles were covered with black cloth to exclude light. The plants were illuminated 14 hr a day. After nine days, the leaves were har-

### TABLE 1

<table>
<thead>
<tr>
<th>Nucleic acid</th>
<th>Counts/min/μg RNA</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsomal RNA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMV-RNA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expt. 1, plants were grown in nutrient solution containing 0.6 μc Pi32 per ml; Expt. 2, plants were grown in nutrient solution containing 0.5 μc Pi32 per ml. Rest of the conditions are described in the text.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### TABLE 2

<table>
<thead>
<tr>
<th>Nucleotides</th>
<th>5'-(3'-)</th>
<th>Expt. 1</th>
<th>5'-(3'-)</th>
<th>Expt. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytidylic acid</td>
<td>224</td>
<td>237</td>
<td>218</td>
<td>235</td>
</tr>
<tr>
<td>Adenylic acid</td>
<td>243</td>
<td>223</td>
<td>232</td>
<td>231</td>
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<tr>
<td>Guanylic acid</td>
<td>235</td>
<td>224</td>
<td>215</td>
<td>216</td>
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<tr>
<td>Uridylic acid</td>
<td>235</td>
<td>218</td>
<td>221</td>
<td>232</td>
</tr>
<tr>
<td>Average</td>
<td>235</td>
<td>226</td>
<td>222</td>
<td>228</td>
</tr>
</tbody>
</table>

* Counts/min/m mole nucleotide. Expt. 1, plants were grown in nutrient solution containing 0.6 μc Pi32 per ml; Expt. 2, plants were grown in nutrient solution containing 0.5 μc Pi32. Rest of the experimental conditions are described in the text.
vested, and the P32-TMV-RNA and P32-microsomal RNA were isolated according to the procedures described below.

**Determination of the radioactivities of microsomal RNA and TMV-RNA:** Unless otherwise stated, all operations were conducted at 0–4°C. Immediately after harvesting the radioactive tobacco leaves, their mid-ribs were removed, and the leaf blades were quickly cut into small pieces. Two gm portions were placed in a homogenizer tube of 15 ml capacity, fitted with a teflon pestle. The leaves were ground for 5 min with 5 ml of 0.5 M sucrose containing 0.01 M tris-HCl buffer at pH 8.0 and 0.002 M MgCl2. The ground material was centrifuged for 20 min at 20,200 g in the refrigerated Servall centrifuge. The residue was washed twice with 3 ml of above grinding medium. The combined supernatants were centrifuged for 2 hr at 105,000 g in the No. 40 rotor of the Spinco Model L ultracentrifuge. After draining the supernatant thoroughly, the pellet was suspended in 1.5 ml of 0.1 M sodium phosphate buffer at pH 7.4 containing 4 x 10⁻³ M EDTA and centrifuged for 20 min at 12,100 g in the refrigerated Servall centrifuge. The residue was extracted once with 1.5 ml of the above phosphate buffer.

To the above combined extracts were added 3 ml of chloroform and 0.06 ml of octanol. The mixture was shaken at room temperature for 15 min and centrifuged in the clinical centrifuge for 10 min at top speed. The top aqueous layer, which was pale green and opalescent, containing TMV and microsomal RNA, was drawn out with a capillary pipette and centrifuged for two hr at 105,000 g in the No. 40 rotor of the Spinco Model L ultracentrifuge. The microsomal RNA, present in the supernatant, and the TMV, present in the pellet, were further purified as described below.

The ratio of light absorption at 280 μm to 260 μm of the supernatant was 0.48 in 0.1 M sodium phosphate buffer at pH 7.4. To this was added 0.3 ml of TMV antiserum (1 mg protein). After 15 min at 0°C, the mixture was centrifuged for 15 min at 7,900 g in the refrigerated Servall centrifuge. There was a small amount of precipitate, which was noticed even with the extracts obtained from healthy tobacco leaves, at the bottom of the centrifuge tube. To the supernatant was added 0.66 ml of 40% TCA with mixing. After 15 min the mixture was centrifuged for 10 min at 7,900 g in the refrigerated Servall centrifuge. The precipitate was washed three times with 1 ml of cold 6% TCA and twice with absolute alcohol. The precipitate was finally washed once with ether at room temperature and air-dried.

The pellet containing TMV was extracted twice with 0.5 ml of 0.1 M sodium phosphate buffer at pH 7.4 containing 4 x 10⁻³ M EDTA. The supernatants, obtained by centrifugation for 15 min at 12,100 g in the refrigerated Servall centrifuge, were combined. To the combined extracts were added 0.1 ml of pancreatic RNAase (400 μg) and a drop of chloroform to prevent microbial growth. The mixture was incubated at room temperature for 24 hr. At the end of the incubation period, the tube was transferred to an ice bath and to this was added with mixing 0.6 ml of cold 40% TCA. After 15 min, the precipitate was recovered by centrifugation. It was washed and air-dried as described above.

The above air-dried precipitates containing microsomal RNA and TMV-RNA were dissolved in 0.3 ml of NaOH, incubated at 30°C for 24 hr, and neutralized with 2.0 N HCl. 0.4 ml aliquots of the neutralized hydrolysates were placed on Whatman No. 3 MM filter papers as narrow bands (10 cm long) and dried in a current of air at room temperature. The chromatograms were developed in the isopropanol-water-ammonia solvent system for 24 hr, dried at room temperature, and the nucleotides were located under ultraviolet light. In this solvent system the alkali-hydrolyzed RNA gives two bands, one containing guanosine 2'-3'-phosphate and the other containing a mixture of 2'-3'-phosphates of adenosine, cytidine, and uridine. These two bands were eluted together by running distilled water through them for 4 hr. A blank filter paper of the same dimensions, which was cut out of Whatman No. 3 MM filter paper, subjected to the above chromatographic procedure, was likewise eluted. The eluates were made up to a suitable volume, and their absorptions were measured in the Beckman spectrophotometer at 260 μm. A known amount of TMV-RNA, subjected to the above procedure, was used as a standard in calculating the RNA contents of the samples.

Aliquots of eluates containing the microsomal RNA and TMV-RNA nucleotides were dried on metal planchetts and their radioactivities were measured.

**Preparation of P32-labeled TMV and TMV-RNA:** Unless otherwise stated, all operations were carried out at 0–4°C. Fifty gm of radioactive leaves were frozen immediately after harvesting.
The frozen material was placed in a large mortar and quickly powdered with a pestle. To the powdered material were added 10 gm of sand and 50 ml of 0.1 M sodium phosphate buffer at pH 7.4 containing \(4 \times 10^{-3} \) M EDTA. The mixture was ground for 5 min; to this were added 100 ml of the above buffer, and the grinding was continued for another 5 min. The ground material was centrifuged for 15 min at 17,300 g in the refrigerated Servall centrifuge. The residue was washed once with 50 ml of the above grinding buffer. The combined supernatants were centrifuged for 1 hr at 78,480 g in the No. 30 rotor of the Spinco Model L ultracentrifuge. The pellets were frozen for 30 min at \(-20^\circ\text{C}\) and then they were soaked overnight at 4° covered with phosphate buffer used for grinding. The virus was isolated from the combined extracts by four cycles of differential centrifugation; the low speed cycles consisted of a 15-min centrifugation in the refrigerated Servall at 17,300 g, and the high speed cycles consisted of a 1-hr centrifugation at 105,000 g in the No. 40 rotor of the Spinco Model L ultracentrifuge. The pellets were always taken up in the phosphate buffer used for grinding the leaves and they were allowed to go into solution gradually. The final preparation, which was colorless and opalescent, was dialyzed for 24 hr against two changes of distilled water (4 liters) with continuous stirring. The yield of the virus was about 120–140 mg.

TMV-RNA was prepared from the purified TMV according to the procedure previously described.\(^4\)

**Determination of specific radioactivities of mononucleotides obtained by hydrolysis of \(^{32}P\)-TMV-RNA with alkali:** One mg of \(^{32}P\)-TMV-RNA was dissolved in 0.5 ml of \(N\) NaOH, incubated at 30° for 24 hr, and neutralized with 2 \(N\) HCl. The rest of the procedure involving chromatography of the neutralized hydrolysate in isopropanol-water-ammonia solvent system, elution of nucleotides from the chromatogram, was exactly the same as described above.

The eluate from the chromatogram containing all four nucleotides was taken to dryness over anhydrous CaCl\(_2\) in a vacuum desiccator at room temperature, and the residue was taken up in 0.05 \(M\) ammonium formate buffer at pH 3.4. A sample of 0.05 ml was placed as a narrow band at a distance of 10 cm from one end of the strip (9 x 55 cm) of Whatman No. 3 MM filter paper, which was wetted with 0.05 \(M\) ammonium formate buffer at pH 3.4 and a current of 10 ma. This procedure separates the mixture of nucleotides into four bands: cytidine 2'-(3')-phosphate, adenosine 2'-(3')-phosphate, guanosine 2'-(3')-phosphate, and uridine 2'-(3')-phosphate. The paper strip was dried at room temperature. The bands were located under ultraviolet light, cut out, and eluted with 0.01 \(N\) HCl for 18–20 hr at room temperature. The amount of nucleotide present in each eluate was determined spectrophotometrically. An aliquot of each eluate was dried on a metal planchet, and the radioactivity was measured.

**Determination of specific radioactivities of mononucleotides obtained by hydrolysis of \(^{32}P\)-TMV-RNA with snake venom phosphodiesterase:** The snake venom phosphodiesterase used in these studies and the conditions of hydrolysis were the same as those of Sugiyama and Fraenkel-Conrat.\(^4\) 0.3 mg of \(^{32}P\)-TMV-RNA was dissolved in 0.06 ml of 0.1 \(M\) borate buffer at pH 8.6 containing 0.005 \(M\) MgCl\(_2\). To this solution were added three 0.02 ml aliquots (0.3 unit/0.02 ml) of snake venom phosphodiesterase at 0, 2, and 4 hr. The hydrolysis was carried out for 6 hr at 37°.

0.06 ml of the hydrolysate was subjected to electrophoresis at pH 3.4 for 3 hr according to the procedure described above. Under these conditions four nucleoside 5'-'phosphates present in the hydrolysate separate in the same order as the nucleotides in the alkali-hydrolysates. The nucleotides were eluted from the electrophoretic strip, and their amounts and radioactivities were determined as described above.

**Determination of the radioactivities of the dialyzable fragments formed during the degradation of \(^{32}P\)-TMV-RNA with snake venom phosphodiesterase:** A reaction mixture containing 1 mg of \(^{32}P\)-TMV-RNA in 0.5 ml of 0.05 \(M\) borate buffer at pH 8.6, containing 0.0025 \(M\) MgCl\(_2\) and 0.7 unit (0.05 ml) of snake venom phosphodiesterase, was dialyzed at 35° against 3 ml of above buffer containing MgCl\(_2\). At 1-hr intervals the dialysis bag was transferred to a tube containing a fresh amount of 3 ml of the above buffer. The hydrolysis was continued for 14 hr with gentle shaking. The absorbancies of dialyzable fragments were measured at 260 ma in the Beckman spectrophotometer, and their radioactivities were measured by drying a known amount on metal planchets. The material present in the dialysis bag at the end of the reaction period was made up to 3 ml
with the above borate buffer, and its absorbancy at 260 m\textmu and its radioactivity were determined.

Reagents: Pancreatic RNA\textasciitilde was purchased from the Worthington Biochemical Corp., Freehold, New Jersey, and sea sand from Merck and Co., Inc., Rahway, New Jersey. P\textsubscript{32} was purchased from the Oak Ridge National Laboratory. Snake venom phosphodiesterase was kindly supplied by Dr. M. Laskowski, Sr., Marquette University Medical School, Milwaukee. TMV antiserum was kindly supplied by Dr. A. Siegel of the University of Arizona.

Results.—Specific radioactivities of microsomal RNA and TMV-RNA: The results presented in Table 1 show that the incorporation of P\textsubscript{32} into microsomal RNA and TMV-RNA was significantly different. The small amount of radioactivity present in the microsomal RNA might represent a new synthesis of microsomal RNA or some species of RNA (template?) occurring associated with the microsomes. Experiments are in progress to elucidate this point. The amount of isotope incorporated into TMV-RNA was greater than that associated with microsomal RNA and this is not surprising because of its extensive synthesis in the cell following infection.

In the previous paper\textsuperscript{e} evidence for the degradation of microsomal RNA following infection with TMV and utilization of its degradation products in the synthesis of TMV-RNA, was presented. If the phosphate moiety of TMV-RNA is derived entirely from the microsomal RNA, its specific radioactivity would have been the same as that of microsomal RNA. Since this is not the case, two possibilities concerning the source of its phosphorus can be suggested: one, the phosphate moiety of TMV-RNA is not derived from the microsomal RNA; and two, the phosphate moiety of TMV-RNA is partly derived from the microsomal RNA and partly from other sources. The latter possibility would give rise to nonuniformity in the distribution of P\textsubscript{32} label in TMV-RNA. The evidence obtained from three different experiments (see below) suggests the remarkable uniformity in the labeling of the entire TMV-RNA molecule.

Uniformity in the labeling of P\textsubscript{32}-TMV-RNA: The results presented in Table 2 show that the radioactivities of nucleoside 2'-(3')-phosphates, obtained by hydrolysis of TMV-RNA with alkali, are similar. Since the synthesis of polyribonucleotides is accomplished by the polymerization of ribonucleoside 5'-phosphate moieties of ribonucleoside 5'-triphosphates, the phosphates appearing as the 2'-and 3'-nucleotides, obtained by alkali hydrolysis, could bear no metabolic relation with its associated base. For this reason, P\textsubscript{32}-TMV-RNA was hydrolyzed with snake venom phosphodiesterase to obtain ribonucleoside 5'-phosphates, and the results presented in Table 2 show that the radioactivities of four nucleotides obtained in this manner are also similar. These results are at variance with those reported by Staehelin,\textsuperscript{f} who observed heterogeneity in the distribution of radioactivity in the ribonucleoside 5'-phosphates. Further evidence in support of the uniformity in labeling of the entire TMV-RNA molecule is presented in Figure 1.

Since snake venom phosphodiesterase hydrolyzes nucleic acid stepwise\textsuperscript{g} from the 3'-hydroxyl end of the chain,\textsuperscript{h} it was thought that the degradation of P\textsubscript{32}-TMV-RNA with this enzyme might give further information concerning the distribution of P\textsubscript{32} label. The absorbancies and the radioactivities of the dialyzable fractions obtained as a result of digestion of P\textsubscript{32}-TMV-RNA with snake venom phosphodiesterase are given in Figure 1. The absorbancy curve is almost superimposed on the radioactivity curve, suggesting the remarkable uniformity in the labeling
throughout the molecule. In 14 hr about 90 per cent of the RNA was rendered dialyzable. The specific radioactivity of the material that remained in the dialysis bag was the same as that of the dialyzable fractions, obtained throughout the experimental period.

Discussion.—As shown in this paper, the radioactivity of TMV-RNA is more than four times that associated with the microsomal RNA, and it is uniformly distributed throughout the entire molecule. Hence, the phosphate moiety of TMV-RNA is not contributed by the microsomal RNA. The evidence for the degradation of microsomal RNA following infection with TMV and the utilization of its degradation products in the synthesis of TMV-RNA was presented in the previous paper, the uridine moiety of the microsomal RNA was indeed utilized in the synthesis of TMV-RNA. On the basis of the results presented in this paper and the previous paper, it can be concluded that only the ribonucleoside moieties of microsomal RNA are utilized in the synthesis of TMV-RNA.

The degradation of microsomal RNA might be effected by the combined actions of ribonuclease and phosphatase present in the tobacco leaf. In addition to the RNAase, the properties of which were described previously, we have evidence for the occurrence of another nuclease in tobacco leaves. The partial purification and properties of phosphatase present in tobacco leaves were previously described. By the action of these three enzymes, microsomal RNA could be degraded to ribonucleosides, which after phosphorylation to nucleoside 5'-triphosphates could serve as substrates for the polymerizing enzyme involved in the synthesis of TMV-RNA. The mode of conversion of ribonucleosides to nucleoside 5'-triphosphates is being studied, and the results will be published elsewhere.

Summary.—The radioactivities of TMV-RNA and microsomal RNA, isolated from infected tobacco leaves grown in the presence of P32, were determined. The specific radioactivity of TMV-RNA is more than four times that associated with the microsomal RNA. The specific radioactivities of four mononucleotides, obtained by the digestion of P32-TMV-RNA with alkali and snake venom phosphodiesterase, are similar. Furthermore, the distribution of radioactive label in the entire molecule of TMV-RNA is remarkably uniform. Hence, the phosphate moiety of TMV-RNA is not derived from the microsomal RNA. On the basis of the results presented in this paper and the previous publication of this series, it is concluded that only the ribonucleoside moieties of microsomal RNA are utilized in the synthesis of TMV-RNA.
The effect of acriflavine on photoreversal of lethal and mutagenic damage produced in bacteria by ultraviolet light*  

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State University of New York, Downstate Medical Center, Brooklyn  

Communicated by M. Demerec, July 25, 1963  

Mutations to prototrophy, induced in auxotrophic bacteria by far ultraviolet light (UV), may be eliminated by subsequent exposure to near UV or visible light (photoreversal), or, in the dark, by temporary suppression of postirradiation protein synthesis ("dark repair"). It has been proposed that photoreversal and "dark repair" of these mutations involve alternative mechanisms for the enzymatic repair of the same premutational damage produced by UV in the bacterial deoxyribonucleic acid (DNA). Since killing by UV is photoreversible, but not subject to "dark repair" (in Escherichia coli B/r), and since the same is true of certain UV-induced mutations other than those to prototrophy, it seems certain that UV produces at least two kinds of photoreversible lesions in bacteria, only one of which is also subject to "dark repair." 

Acriflavine has been shown to interfere with "dark repair" of UV-induced prototrophy, and it has been suggested that it does so by combining with and modifying the irradiated bacterial DNA in a way that reduces the accessibility of the premutational lesions to the postulated "dark repair" enzyme. If the lesions leading to prototrophy are indeed different from those leading to death, acriflavine might be expected to interfere also with photoreversal of these mutations, but not necessarily with photoreversal of killing. In this report, experiments are described in which the effect of acriflavine on photoreversal of both lethal and mutagenic...