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29 May 1962

Biosynthesis of Infectious Tobacco Mosaic Virus Ribonucleic Acid in a Cell-Free Medium

Abstract. When cell-free preparations containing large molecules and subcellular bodies extracted from tobacco-leaf cells infected with tobacco mosaic virus were incubated with $MgCl_2$ and the four ribonucleoside-5′-triphosphates, the level of infectivity increased about threefold in 30 minutes. The mechanism synthesizing the new infectivity was believed to be located within isolated nuclei. Ultrasonic rupture of the nuclear membranes appeared to increase the rate of synthesis.

Evidence for the existence of naturally occurring infectious ribonucleic acid (RNA) of tobacco mosaic virus in infected plant cells has been reported by several virologists (*1*). Data presented by Engler and Schramm (*2*) suggested that this is the first-formed infectious stage of the virus. Recent success in the biosynthesis of RNA from its nucleoside-5′-triphosphates (*3*) suggested to us that similar techniques might be applied to the synthesis of infectious viral RNA (*4*).

We assumed that a synthesizing mechanism might be isolated from infected plant cells which would be capable of combining adenosine triphosphate, guanosine triphosphate, uridine triphosphate, and cytidine triphosphate in the proper linear sequence to form infectious viral RNA.

We knew that the presence of ribonuclease in the synthesizing system could destroy the newly formed viral RNA, and we assumed that any synthesizing mechanism isolated from infected cells would be as large as, or larger than, the RNA being formed.

Gel filtration techniques in which 2-percent particulate agar gels were used, were devised for isolating the synthesizing mechanism in a ribonuclease-free state. (It had been determined that the pores of 2-percent agar excluded tobacco mosaic virus RNA while admitting smaller molecules.) Agar gel particles prepared by spraying hot agar through cold atmospheres or by chopping solidified agar were sized by passage through 24-mesh screens and retention on 48-mesh screens. Slurries of the agar particles were allowed to settle in chromatographic tubes to give columns 40 to 60 cm high, which were then conditioned by passage of isotonic sucrose buffered at *p*H 7.2 (0.25 mole of sucrose, 0.003 mole of $CaCl_2$, and 0.006 mole of tris).

Unstable tobacco mosaic virus RNA, phenol-prepared, was used to inoculate the leaves of Turkish or Havana 38 tobacco so that there would be no external carry-over of inoculum that might complicate interpretation of the experimental results. After the desired infection time had elapsed (14 hours or longer), the leaves were placed in a plier-type juice extractor and pressed so that the extracted sap fell directly on the top of the gel columns. Approximately 40 g of leaf tissue were extracted in each experiment. The applied sap was driven through the columns by the application of additional sucrose buffer. The downward migration of the green chloroplasts served as a visual indicator of the movement of the large molecules and subcellular bodies. Collection of the effluent was begun just prior to the elution of the chloroplasts and was continued in most experiments until 100 to 150 ml had been collected.

Extensive studies with the light microscope showed that the effluent contained chloroplasts, starch grains, nuclei, and many smaller particles, but no structures as large as cells were seen. It was noted that a green zone always remained at the top of the gel column in each experiment. When samples from these zones were studied, single cells and groups of cells were observed. It was also noted that our 2-percent agar columns would not pass a single-celled alga considerably smaller than tobacco leaf cells. All of these observations suggested that the gel columns were filtering out intact cells to give only cell-free extracts.

The movement of a brown-colored zone down the column indicated the migration rate of the smaller molecules. Collection of effluent was always stopped before this zone was eluted.

Low-speed centrifuging (4000*g* for 15 minutes) was used to concentrate the synthesizing mechanism in the effluent while discarding most of the free tobacco mosaic virus and viral RNA. The green-colored pellets were usually resuspended in 4 to 6 ml of sucrose buffer containing 0.003 mole of K_2HPO_4 and 0.01 mole of $MgCl_2$. The suspension was then divided into two equal parts, of which one, the control solution, received no further treatment and the other, the chemically treated solution, received 1-mg amounts of the four ribonucleoside-5′-triphosphates. The tubes were usually incubated side by side for 1 hour, samples being taken from each tube at 10-minute intervals and inoculated on opposite halves of 24 to 36 pinto bean leaves for comparative assay of levels of infectivity. Counts of the necrotic local lesions produced indicated the effectiveness of the induced biosynthesis.

The first experiment, conducted on 12 February 1962, suggested that synthesis of viral RNA was occurring in the chemically treated solution. The results of this experiment are presented in Table 1. Assay showed the level of virus in the chemically treated solution to be 184 percent higher than the level in the control solution. Numerous subsequent experiments, with slight modifications of the technique described, were completed, with similar results. Usually, no differences could be found at zero time, slight synthesis was evident at 10 minutes and more at 20 minutes, and synthesis was maximum after 30 minutes of incubation. Amino acids and penicillin G were added in some experiments and did not appear to influence the rate of synthesis.

In one experiment virus protein and C^{14}-labeled adenosine triphosphate were mixed with the virus-synthesizing extract before it was divided into control and treated fractions. The three other nucleotides and $MgCl_2$ were added to the treated solution. After incubation for 30 minutes at 25°C the synthesizing mechanisms were removed by centrifuging and resuspended in a second

lot of incubation medium (control and treated) for a second 30-minute incubation period. The procedure was repeated for a third incubation period with a final sedimentation. The clarified media from all incubations were stored for 20 hours at 2°C to allow virus protein to coat the newly synthesized viral nucleic acid. The media were diluted tenfold prior to the assay for infectivity and were subjected to repeated cycles of low- and high-speed centrifugation to eliminate materials of low molecular weight, including unincorporated adenosine triphosphate.

The results of the experiment are presented graphically in Fig. 1. The amount of viral RNA synthesized by the controls was apparently related to the amount of the naturally occurring reserves of nucleotides present in the synthesizing mechanism. In the three successive incubations the level of infectivity in the control changed from 1297 to 563 to 164. In contrast to this, the net value for synthesis of viral RNA, as represented by the differences between the infectivities of the control and of the treated lots, remained fairly constant, changing only from 434 to 209 to 349 in the successive incubations.

The agreement between the radioactivity and the infectivity data is probably the result of the specificity of the tobacco mosaic virus protein in selectively coating the viral RNA and the specificity of the centrifuge techniques in isolating the tobacco mosaic virus rods. This agreement indicates that viral RNA was indeed synthesized in the test tubes. Radioactive adenosine triphosphate of small molecular weight was built into infectious viral RNA of high molecular weight, which was coated by virus protein to make infectious nucleoprotein virus rods that were radioactive. Both the infectivity and the radioactivity data indicate that viral RNA was synthesized repeatedly by the same mechanism.

In another experiment the virus-synthesizing mechanism was retained by a 5-μ filter. Infective material which passed through the 5-μ filter but which would not pass through a 0.45-μ filter was always adversely affected by the ribonucleotide treatments and apparently had an incomplete synthesizing mechanism. The only structures within tobacco cells that are 5 μ in diameter or larger are the nuclei and the chloroplasts. In additional experiments when nuclei and chloroplasts from infected leaves were separated by sucrose den-

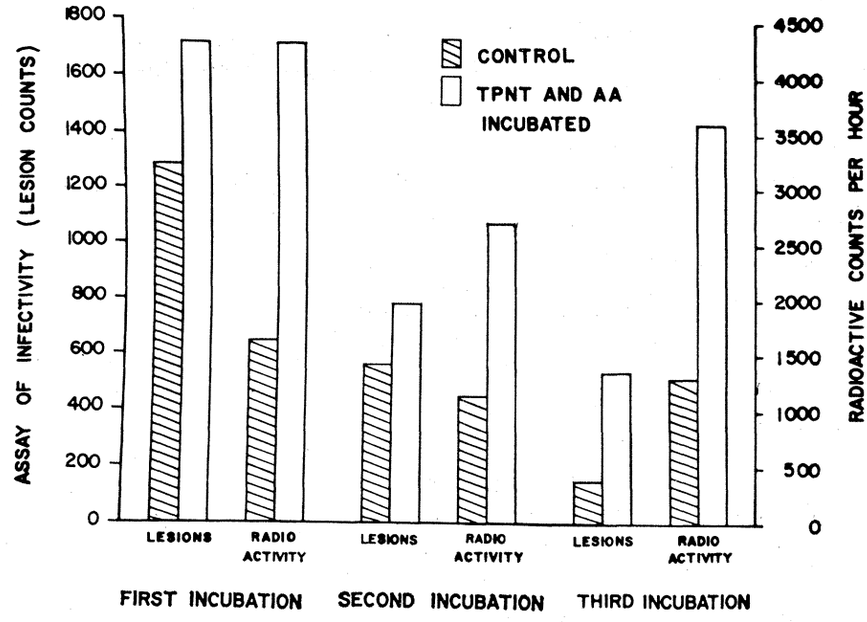

Fig. 1. Repeated biosynthesis of viral RNA by the same synthesizing cell-free system with incorporated C^{14}-labeled adenosine triphosphate in the presence of tobacco mosaic virus protein. The tobacco mosaic virus nucleoprotein was purified by differential centrifugation prior to counting for radioactivity. *AA*, Amino acid; *TPNT*, triphosphate nucleoside.

Table 1. Biosynthesis of infectious tobacco mosaic virus RNA in a cell-free medium by a synthesizing mechanism extracted from leaves of Turkish tobacco infected with the viral RNA. The basic incubation medium (*p*H 7.2) contained (in moles) sucrose (0.25), $CaCl_2$ (0.003), tris buffer (0.006), K_2HPO_4 (0.003), $MgCl_2$ (0.01), and penicillin G (0.002). Duration of the original infection in tobacco was as follows: experiments 1 through 4, 14 hours; experiment 5, 28 hours; experiment 6, 52 hours.

Experiment (No.)	Period of incubation at 25°C (min)	Assay of infectivity* (number of lesions)		Increase over control (%)
		Control	Treated	
		Triphosphate nucleosides†		
1	60	77	219	184
2	70	11	38	245
3	10	380	385	1
3	20	283	561	98
3	30	319	817	156
3	40	444	877	98
3	50	299	600	101
		Triphosphate nucleosides and amino acids‡		
4	0	25	18	−28
4	10	5	27	440
4	20	6	15	150
4	30	14	20	42
4	40	9	21	133
4	50	6	17	183
4	60	7	35	400
5	10	107	193	80
5	20	97	306	215
5	30	72	297	313
5	40	77	156	102
5	50	105	182	73
5	60	76	115	51
6	0	41	40	−2
6	10	50	96	92
6	20	35	106	203
6	30	41	130	217
6	40	48	99	106
6	50	52	108	108
6	60	21	99	371
6	70	15	53	253

* In experiment No. 1, assay was on 61 *Nicotiana glutinosa* leaves; in all the other experiments assay was on pinto beans. The half-leaf comparison method was used, with 24 to 36 replications per treatment. † The triphosphate nucleosides adenosine triphosphate, uridine triphosphate, guanosine triphosphate, and cytidine triphosphate were added in 1-mg amounts, at zero time in the incubation. Noninfectious tobacco mosaic virus protein was added to the extracted synthesizing mechanism before division into control and treated lots. ‡ The triphosphate nucleosides were added in 1-mg amounts at zero time in the incubation; 1 mg of each of the 16 amino acids of tobacco mosaic virus protein was also added.

sity gradient centrifugation, only the nuclear fraction was able to synthesize viral RNA. Other published evidence has suggested that the nuclei are the site of viral RNA synthesis (*5*).

We have also found that the effectiveness of the nuclear fraction in synthesizing viral RNA can be greatly enhanced if the nuclear membranes are ruptured by ultrasonic treatments. In one experiment synthesis jumped from 10 percent to 116 percent above that of the control when the membranes were ruptured.

Preliminary reports on the biosynthesis of viral RNA described in this report have been published elsewhere (*6*). Our conclusions regarding such biosynthesis in a cell-free medium have been confirmed in experiments reported by Kim and Wildman (*7*).

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24 September 1962

Tritium and Helium-3 in Solar Flares and Loss of Helium from the Earth's Atmosphere

Abstract. Analysis of the data gathered by the Discoverer XVII satellite on the constituents of solar flares leads to results that have broad implications in geophysics and solar physics.

It has recently been suggested that H^3 and He^3 may be produced in solar flares as well as the surfaces of magnetic stars by bombardment of He^4 with accelerated protons (*1*). At present, the only direct evidence of such stellar processes is the discovery of H^3 and He^3 in the casing of the earth satellite Discoverer XVII which was flown following the solar flare of 12 November 1960 (*2, 3*). The amounts of H^3 and He^3 recovered from the satellite are two orders of magnitude too large to be accounted for by spallation of the casing material. It was concluded that the solar flare radiation intercepted by the satellite consisted of 0.4 percent H^3, 10 percent He^3, and 90 percent $H^1 + He^4$ with errors of at least a factor of three (*2, 3*). Despite these large errors, the composition of the radiation detected by Discoverer XVII has broad implications in geophysics and in solar physics: (i) The greater part of the He^3 and a significant fraction of the H^3 in the earth's atmosphere probably is accreted from the sun. (ii) Escape of helium from the atmosphere most probably is thermally controlled. (iii) Cross sections for H^3 and He^3 production by $He^4(p,2p)H^3$, $He^4(p,pn)He^3$, and $He^4(p,d)He^3$ reactions near the threshold energies are consistent with the ratio of the two nuclides in the flare radiation, indicating that H^3 and He^3 were indeed produced by these reactions rather than by fusion or by spallation of heavier nuclei in the sun. (iv) The overall composition of the radiation can be reproduced by the solar flare model of Gold and Hoyle (*4*).

Table 1. Threshold energies and total cross sections for production of H^3 and He^3 by 28-Mev protons on He^4.

Reaction	Energy (Mev)	Cross section (mbarn)
$He^4(p,2p)H^3$	25.0	8.9 + 1.0
$He^4(p,pn)He^3$	25.5	4.8 + 1.3
$He^4(p,d)He^3$	23	50

1) Measurements of the flux of solar flare particles of energy above 30 Mev at the top of the earth's atmosphere appear to be converging on a global average annual value of the order of 100 per cm^2-sec (*5, 6*). If we assume the composition of the 12 November radiation to be typical of solar flares, we obtain a global average annual influx of 10 He^3 atoms and 0.4 H^3 atoms per cm^2-sec. Spallation of air molecules by the solar protons or interactions of secondary neutrons with nitrogen (*6, 7*) produce H^3 at a rate comparable to the rate of accretion. The only other significant source of H^3 is the production by galactic cosmic rays, estimated at 0.1 to 1.0 atom per cm^2-sec (*7, 8*). Since the rate of He^3 production by spallation is comparable to the rate of H^3 production (*9*), we have about 2 He^3 atoms per cm^2-sec produced in the earth's atmosphere as compared to 10 atoms per cm^2-sec accreted from the sun.

2) Setting the rate of escape of He^3 from the atmosphere equal to the rate of accumulation, and taking the number of He^3 atoms per square centimeter column of the atmosphere as 1.5×10^{14}, we arrive at a mean residence time:

$$\tau\,(He^3) = \frac{1.5 \times 10^{14}}{12 \times 3 \times 10^7} = 4 \times 10^5 \text{ yr}$$

The atmospheric residence time of He^4, calculated from the decay of uranium and thorium in rocks and the atmospheric concentration, is greater than 2×10^6 years and probably of the order of 10^7 years (*10*). Such a marked difference between the He^3 and He^4 residence times gives an important clue to the mechanism by which helium escapes from the earth; a mechanism strongly dependent on mass is required. Of the various models proposed, those in which the escape rate is controlled by diffusion or by collision or photochemical processes fail to meet this requirement. Thermal escape processes (*11*), on the other hand, are controlled by a Boltzmann factor involving the gravitational energy exponentially, and consequently they discriminate strongly against heavier isotopes. We find, for several model atmospheres (*11, 12*) in which thermal control of the escape rate is assumed $\tau(He^3) = 4 \times 10^5$ years and $\tau(He^4) = 9 \times 10^6$ to 2×10^7 years at temperatures of 1900° to 2100°K. Temperatures as high as 2100°K have been calculated from satellite drag at high altitudes and latitudes during periods of high solar activity (*13*).

The apparent negative correlation between the sunspot cycle and the amount of H^3 deposited in Greenland snows (*14*) is not at variance with our conclusion that significant amounts of H^3 are contributed to the earth's atmosphere by solar flares. Tritons entering the atmosphere with energies below 500 Mev, as well as tritons produced in the atmosphere by solar protons (*15*), are stopped at altitudes well above the polar tropopause. On the other hand, some 20 percent of the H^3 produced in polar regions by galactic cosmic rays is formed in the troposphere (*7*). Studies of the distribution of radioactive fallout from