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Production of Mutants of Tobacco Mosaic Virus by Chemical Alteration of its Ribonucleic Acid *in vitro*

WHILE many agents are known to act as mutagens on the living cell, there have been no convincing reports that the genetic character of isolated particles, such as viruses or transforming principles, have been changed *in vitro*. Recent results with tobacco mosaic virus help to overcome this difficulty. The infective unit of the virus is its ribonucleic acid component, which can be isolated in active form[1]. It is made up of 6,000 nucleotides that probably form a single chain[2].

Recently, Schuster and Schramm[3] have used nitrous acid to convert adenine, guanine and cytosine into hypoxanthine, xanthine and uracil, respectively, while the ribonucleic acid strand remains intact. It was shown that the alteration of any one of about 3,000 nucleotides is lethal. Possibly, changes of other bases are mutagenic.

On the basis of this work we studied a simple symptom of mutation, the production of necrotic lesions on a tobacco variety (Java) on which the untreated strain produces chlorotic lesions only.

Table 1. MUTAGENIC EFFECT OF NITROUS ACID ON RIBONUCLEIC ACID. 2 vol. of 0·19 per cent ribonucleic acid extracted from tobacco mosaic virus *vulgare* by phenol (ref. 1) were mixed with 1 vol. 4 *M* sodium nitrite and 1 vol. 1 *M* acetate *p*H 4·8 at 22° C. After time *t*, the ribonucleic acid is diluted to 19 μgm./ml. with *M*/15 phosphate, and assayed. Control dilutions of untreated ribonucleic acid contain an equivalent amount of nitrite. Time τ (equation 1) is 18·5 min. Total infections are given as numbers of lesions (per leaf) on Xanthi, and as the sum of chlorotic and necrotic lesions on Java; mutant infections as numbers of necrotic lesions per leaf on Java

t (min.)	*t*/τ	Total infections		Mutant infections	Mutant infections (per cent)
		Xanthi	Java	Java	Java
1	0·05	72·8	183	1·4	0·8
4	0·22	72·4	130	2·5	1·9
8	0·43	89·8	188	4·5	2·4
16	0·86	59·3	97	5·4	5·6
32	1·73	36·1	63	6·6	10·5
64	3·46	10·4	21	2·1	9·8
96	5·2	2·7	3·5	0·5	15·5
Untreated controls, μgm./ml. ribonucleic acid					
19		155	138	0·3	0·2
1·9		31·2	42	0·1	0·3
0·19		3·1	10	0	—
× 0·019		0·3	0·8	0	—

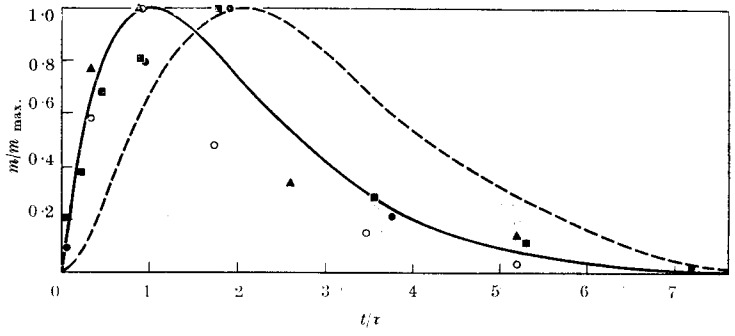

Fig. 1. Dependence of the concentration of mutants (number of necrotic lesions on Java tobacco, relative to maximum value) on the time of incubation with nitrous acid, related to τ. ———, Single-hit curve (equation 3); – – –, double-hit curve; ▲, tobacco mosaic virus *vulgare* (assayed at 0·7 μgm./ml.); ●, ribonucleic acid from tobacco mosaic virus *vulgare* (assayed at 39 μgm./ml.); ■, ribonucleic acid from tobacco mosaic virus *vulgare* (assayed at 19 μgm./ml.); ○, ribonucleic acid from light-green strain *B*11 (assayed at 9·5 μgm./ml.)

Virus or isolated ribonucleic acid was treated with nitrous acid. After times t, samples were diluted to a constant concentration for assay on Xanthi and Java tobacco plants. The conditions and results are given in Table 1. The total concentration n of infective particles was measured by the number of lesions on Xanthi, and of chlorotic and necrotic lesions on Java tobacco, as compared with standard dilution curves. n decreases exponentially with t:

$$n = n_0 \exp(-t/\tau) \qquad (1)$$

where τ is the average time for one lethal conversion of a base per ribonucleic acid molecule.

The necrotic lesions on Java tobacco are due to mutated particles, and their number is expected to be nearly proportional to their concentration m. If the alteration of a single base is mutagenic, and if the average number of such mutations per ribonucleic acid molecule in the time τ is p, the concentration of such mutants is:

$$m = n_0 p \frac{t}{\tau} \exp(-t/\tau) = np\frac{t}{\tau} \qquad (2)$$

or in terms of the maximum value of m:

$$\frac{m}{m_{\max.}} = e\,\frac{t}{\tau}\,\exp(-t/\tau) \qquad (3)$$

Fig. 1 represents measurements under different conditions with tobacco mosaic virus and with ribonucleic acid derived from two strains. For each case, τ was determined experimentally, using equation (1). The numbers of necrotic lesions on Java tobacco, divided by the maximum value reached in time t, were plotted against t/τ. The experimental results were in agreement with the theoretical relation (3).

The results lead to the following conclusions.

(1) Ribonucleic acid and tobacco mosaic virus treated with nitrous acid give rise to a much larger number of necrotic lesions on Java than untreated material assayed at the same concentration; it is twenty times the spontaneous level when total infectivity has dropped to only half the original value (Table 1). This appearance of mutants at low rates of inactivation excludes selection, and cannot be due to pre-existing mutants, or to mutants arising in the plant.

(2) The mutagenic effect on tobacco mosaic virus and on its isolated ribonucleic acid is equal when the fraction of bases altered in the ribonucleic acid, measured by t/τ, is the same. Also, tobacco mosaic virus treated with nitrous acid has the same proportion of mutants as ribonucleic acid isolated after the treatment. Thus, the mutants are produced by chemical reactions with ribonucleic acid rather than by aggregation phenomena, or by changes in the protein.

(3) The production of mutants is at first linear with t and follows a single-hit curve, irrespective of varying conditions (Fig. 1). Therefore, alterations of single nucleotides are mutagenic.

At $t = \tau$, where one out of about 3,000 nucleotides is altered[3], 6 per cent of the primary infections on Java plants infected with *vulgare* are found to be necrotic (Table 1). If the plating efficiency is assumed to remain unchanged upon mutation, the alteration of any one out of 180 of the total 6,000 nucleotides in the ribonucleic acid strand would be mutagenic, leading to necrotic lesions on Java; this assumption may be subject to later correction.

(4) A variety of other mutants is also produced by nitrous acid. In order to detect them, we isolated individual lesions produced by ribonucleic acid treated with nitrous acid on Xanthi tobacco ($t/\tau = 5{\cdot}2$, Table 1) and assayed them on Java and Samsun plants. Out of 60 lesions, 20 produced no infection, 33 led to a variety of altered symptoms and only 7 gave apparently unchanged symptoms on Samsun tobacco. The altered symptoms proved genetically stable in a transfer experiment. From a control dilution of untreated ribonucleic acid, 64 out of 65 lesions produced infection and no altered symptoms were detected.

We are led to conclude that the chemical alteration of individual bases of the ribonucleic acid molecule *in vitro* is mutagenic. A variety of different mutants may be produced in this way. It remains undecided which of the three types of chemical change caused by the nitrous acid (see above) are mutagenic, and how the chemically altered ribonucleic acid is related to its progeny. In the case of a conversion of cytosine into uracil, it is conceivable that the altered ribonucleic acid undergoes identical reproduction in the host cell.

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