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Some Properties of Infective Preparations made by Disrupting Tobacco Rattle Virus with Phenol

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SUMMARY: Preparations made by treating tobacco rattle virus with phenol were about 5 % as infective as the initial virus suspensions when assayed on French bean, but less on tobacco. Virus nucleic acid seems to be a major constituent of such preparations. By contrast with whole virus, nucleic acid preparations lost infectivity when incubated for 20 min. with 0.02 mg. ribonuclease/l. or when stored for a day at 20°. Nucleic acid preparations contain threads about 1 μ in diameter but very few or no virus rods. The infectivity of nucleic acid preparations was little affected by high-speed centrifugation for periods in which more than 95 % of whole virus was sedimented. Ultraviolet-irradiated nucleic acid preparations were photoreactivable although irradiated preparations of whole virus were not. Nucleic acid preparations were only infective when made from purified virus suspensions or from frozen and clarified saps when these contained infective rod-shaped particles 179-192 μ long. Phenol treatment of purified non-infective particles 73-77 μ long yielded non-infective preparations.

Since the work on tobacco mosaic virus by Fraenkel-Conrat (1956) and by Gierer & Schramm (1956), infective nucleic acid preparations have been obtained from several other plant and animal viruses. Among plant viruses, these preparations have been made from purified samples of tobacco ringspot (Kaper & Steere, 1959) and potato X (Bawden & Kleczkowski, 1959*a*) viruses which have particles of quite different shape and stability from those of tobacco mosaic virus. A feature common to these nucleic acid preparations, whether they are made by treatment with phenol (Gierer & Schramm, 1956), heating with detergent (Fraenkel-Conrat, Singer & Williams, 1957) or heating in buffer (Kaper & Steere, 1959), is that their infectivity is usually less than 1 % of that of the initial virus suspensions and is destroyed by minute amounts of ribonuclease. The present paper describes some properties of nucleic acid preparations made by the phenol method from a second rod-shaped plant virus, tobacco rattle. These preparations seem considerably more infective, relative to whole virus, than those obtained from other viruses.

METHODS

Tobacco rattle virus was 'purified' as described previously (Harrison & Nixon, 1959). Phenol treatment (Gierer & Schramm, 1956) was as follows: 5 ml. tobacco rattle virus suspension (200 mg./l.) in M/60 (pH 7.0) phosphate buffer was vigorously shaken for 2 min. with an equal volume of water-saturated phenol and the mixture then centrifuged for 3 min. at 9000 g. The aqueous top layer was removed from the centrifuge tubes to within 5 mm. of the interface with the bottom layer and shaken with 50 ml. ether in a

separating funnel. The bottom layer of liquid was drawn off, and, in most experiments, the ether treatment repeated. The final preparations are referred to below as 'nucleic acid preparations'. The centrifuge and all solutions were kept at 5° before use. Infectivity was assayed on French bean (*Phaseolus vulgaris* L. var. Prince) or tobacco (*Nicotiana tabacum* L. var. White Burley) (Cadman & Harrison, 1959). 'Super-floss Celite' powder (Johns-Manville Ltd.) was mixed with all inocula.

RESULTS

Stability of infective preparations

Table 1 shows that after allowing for the differing susceptibility of different batches of test plants, the infectivity of different nucleic acid preparations relative to virus was very similar. Although infectivity decreased little in 20 min. at room temperature, only small and variable amounts remained after 20 hr. The variability may possibly be due to the presence of minute but differing amounts of stabilizing or inactivating substances in different preparations; such substances perhaps come from the glassware. At least 10% of the initial infectivity remained after nucleic acid preparations were frozen for 20 hr., and up to half remained after dialysis for 20 hr. at 5° against distilled water or 0.1 M-ammonium acetate. By contrast with nucleic acid preparations, untreated tobacco rattle virus lost little or no infectivity when kept for 20 hr. at room temperature; when mixed with nucleic acid prepara-

Table 1. *Effect of ageing and of ribonuclease on infectivity of nucleic acid preparations or purified virus*

The nucleic acid preparations were made from suspensions containing approximately 200 mg. tobacco rattle virus/l. Thus the virus suspension at 1/500 was at a concentration of 0.4 mg./l.

Inoculum	Dilution	Storage tem- perature	Infectivity*		
			Expt. 1	Expt. 2	Expt. 3
(a) Inoculated after 20 min.					
Nucleic acid	1/2	5°	10,600	10,200	11,400
Nucleic acid	1/20	5°	913	463	—
Nucleic acid	1/2	20°	7,330	11,250	—
Nucleic acid + 0.02 mg./l. ribonuclease	1/2	20°	48	0	6
Virus	1/500	20°	1,000	1,000	1,000
(b) Inoculated after 20 hr.					
Nucleic acid	1/2	-10°	—	1,840	1,000
Nucleic acid	1/2	20°	30	3	248
Virus	1/500	20°	1,000	1,000	1,000
Virus	1/2500	20°	—	121	256
Virus + 0.02 mg./l. ribonuclease	1/500	20°	425	486	1,320
Virus + nucleic acid (at a dilution of 1/2)	1/500	20°	—	122	376

* Infectivity was assayed on eight half-leaves of French bean. The total numbers of lesions counted were multiplied by the factor needed to bring the number for virus diluted 1/500 to 1000.

tions, however, the virus was less stable than in water or phosphate buffer and only about 10 % of its infectivity seemed to remain after 20 hr. at room temperature in this medium. Nevertheless, the stability of whole tobacco rattle virus, measured in this way, was considerably greater than that of the nucleic acid preparations.

Preparations of nucleic acid from tobacco mosaic virus vary in infectivity with dilution in quite a different way from untreated tobacco mosaic virus (Bawden & Pirie, 1957), but with tobacco rattle virus there is little difference in this respect between nucleic acid preparations and virus (Table 2). Reasonably accurate measurements of the infectivity of nucleic acid preparations from tobacco rattle virus relative to that of the virus suspensions from which they came can thus be made. Different preparations have given figures of 2–10 % when assayed on French bean, values of 4–6 % being commonest. The relative infectivity of the nucleic acid preparations, however, depends on the species of plant used for the assays, and values obtained with tobacco are less than those with French bean. For example, nucleic acid and virus inocula that gave 855 and 219 lesions, respectively, on French bean, gave 42 and 69 lesions on tobacco. Thus although French bean was more susceptible to the virus than tobacco, the difference was still greater for the nucleic acid preparations. Unusually great susceptibility to nucleic acid inocula may be a general property of French bean, for Commoner (1957) found that preparations of nucleic acid from tobacco mosaic virus which had a relative infectivity of 0.1–0.5 % when assayed on *Nicotiana glutinosa*, had values of 10 % or more on French bean. Tobacco mosaic virus-nucleic acid produced more lesions on French bean than on *N. glutinosa*.

Table 2. *Effect of dilution on infectivity of virus and nucleic acid preparations*

The nucleic acid preparations were made from suspensions containing 200 mg. virus/l. Thus the virus suspension at 1/100 contained 2 mg. virus/l.

Inoculum	Dilution	Infectivity*	
		Expt. 1	Expt. 2
Nucleic acid	1/2	3040	1580
Nucleic acid	1/10	973	455
Nucleic acid	1/50	167	80
Virus	1/100	2005	694
Virus	1/500	379	132
Virus	1/2500	79	43
% infectivity†	$\frac{\text{Nucleic acid}}{\text{Virus}}$	4.6	6.5

* Total lesions produced on eight half-leaves of French bean.

† Calculated from the dilution required to produce 80 lesions/half leaf.

Effect of ribonuclease

Crystallized pancreatic ribonuclease (Armour Pharmaceutical Co.) at 0.02 mg./l. almost completely abolished the infectivity of nucleic acid preparations in 20 min. at room temperature, whereas at this concentration the

enzyme had little effect on the infectivity of virus after 20 hr. (Table 1). The effect of ribonuclease on infectivity of virus inocula does not increase with time of incubation with the enzyme (Nixon & Harrison, 1959). Crystallized trypsin at 1 mg./l. was less effective in abolishing the infectivity of nucleic acid preparations than ribonuclease at 0.02 mg./l., and its action probably came from traces of ribonuclease still present. Chymotrypsin at 1 mg./l. (provided by Dr A. Kleczkowski), which was free from material able to hydrolyse yeast nucleic acid, had no measurable effect on the infectivity of nucleic acid preparations in 20 min. at room temperature.

Infective nucleic acid preparations can be made direct from tobacco sap which has been frozen and then clarified by low-speed centrifugation (Table 3). Their infectivity is destroyed by 20 min. incubation with ribonuclease (0.02 mg./l.) or by 20 hr. storage at 20°. The relative infectivity of preparations made from sap is, however, much less than those made from purified tobacco rattle virus and is usually about 0.2 % of that of the original sap.

Table 3. *Effect of aging and of ribonuclease on infectivity of sap or of a nucleic acid preparation made from sap*

Infective tobacco sap was stored for 2 days at -10°, then thawed and clarified immediately before use. The nucleic acid preparation from sap was made by shaking with an equal volume of water-saturated phenol; the subsequent procedures were as already described.

Inoculum	Dilution	Storage temperature	Infectivity*	
			inoculated after 20 min.	inoculated after 20 hr.
Nucleic acid	1/1	5°	136	1†
Nucleic acid + ribonuclease (0.02 mg./l.)	1/1.2	20°	1	—
Sap	1/1000	20°	64	67
Sap + ribonuclease (0.02 mg./l.)	1/1000	20°	50	67
Sap	1/5000	20°	—	24

* Total lesions produced on eight half-leaves of French bean.

† Kept at 20°.

High-speed centrifugation

Less than a quarter of the infectivity of nucleic acid preparations was sedimented by high-speed centrifugation in conditions in which over 95 % of the virus was sedimented (Table 4). The material sedimented from nucleic acid preparations seemed not to contain contaminating virus particles, for its infectivity was abolished by ribonuclease (0.02 mg./l.).

Electron microscopy

Nucleic acid preparations sometimes contained a very few virus rods, but comparison with virus preparations of known concentration showed that the rods were too few to account for even 1 % of the observed infectivity. Preparations dialysed for 20 hr. at 5° against distilled water or 0.1 M-ammonium acetate (pH 6.8) were mounted for electron microscopy by spraying on to freshly cleaved muscovite mica and shadowing with platinum. A layer of carbon

was then deposited at normal incidence, followed by direct stripping on to a water surface and mounting on 4- or 7-hole platinum disks. The method is essentially that of Hall (1956), but the collodion and silicon monoxide films are replaced by a single layer of evaporated carbon. Samples dialysed against ammonium acetate contained slender fibrils 4–6 $m\mu$ in diameter, but there was also evidence of considerable mounts of much smaller material, which could be detected only by the alignment of the granules of platinum. These very fine threads appeared to be about 1 $m\mu$ in diameter, and the larger fibrils may well be aggregates of the threads. Samples dialysed against distilled water showed the fine threads but not the fibrils, which were replaced by irregular blobs which might well be more compact aggregates of the threads. Nucleic

Table 4. *Effect of high-speed centrifugation on infectivity of virus and nucleic acid preparations*

Three ml. nucleic acid preparation diluted 1/2, or virus preparation diluted 1/50, in $m/60$ phosphate buffer (pH 7) was centrifuged for 2 hr. at 5° and 47,000 g in a swing-out rotor. Samples were removed by carefully lowering to the required depth a Pasteur pipette whose tip was bent through an angle of 90°. Each sample was split into 2 parts; ribonuclease to final concentration 0.02 mg./l. was added to one part and distilled water to the other. Samples were inoculated approximately 20 min. after making the mixtures.

Position of sample in centrifuge tube	Infectivity*			
	Nucleic acid		Virus	
	Without ribonuclease	With ribonuclease	Without ribonuclease	With ribonuclease
Top 1.2 ml.	1365	1	7	9
Bottom 1.0 ml.	1105	1	35	19
Dregs and re- suspended pellet	877	0	1630	844

* Total lesions produced on 8 half-leaves of French bean.

acid preparations made from tobacco mosaic virus by the phenol method and dialysed against water or ammonium acetate had the same appearance as those from tobacco rattle virus. In forming more compact aggregates in water than in ammonium acetate, the nucleic acid preparations behave like the nucleic acid fibres projecting from the ends of partially degraded rods of tobacco mosaic virus. When mounted in acetate these fibres were up to 2750 $m\mu$ long but when mounted in water they were thicker and much shorter (Hart, 1958). In one experiment a nucleic acid preparation from tobacco rattle virus was dialysed against an ammonium acetate/acetic acid mixture with the pH value (pH 4.8) of the distilled water used previously. This sample appeared very similar in the electron microscope to those suspended in pH 6.8 acetate, indicating that the samples in acetate or distilled water did not differ in appearance because of the difference in pH value. Hart (1958) suggested that the different appearances of nucleic acid fibres prepared in water or acetate may be caused by the effects of these media on the degree to which the fibres coil; this is also a plausible explanation of our observations.

Photoreactivation of ultraviolet-irradiated preparations

Tobacco mosaic and tobacco rattle are the only two plant viruses tested whose residual infectivity after ultraviolet-irradiation is the same whether assayed on plants kept in daylight or in darkness after inoculation; that is, these viruses are not photoreactivable (Bawden & Kleczkowski, 1955; Cadman & Harrison, 1959). Recently, however, Bawden & Kleczkowski (1959*b*) showed that ultraviolet-irradiated *nucleic acid preparations* from tobacco mosaic virus are photoreactivable; Table 5 provides evidence that nucleic acid preparations from tobacco rattle virus are also photoreactivable. The inactivation curve obtained by plotting residual infectivity against dose of ultraviolet radiation was exponential whether or not the assay plants were kept in daylight after inoculation, but when they were kept in darkness its slope was steeper. The ratio of the slopes of the two inactivation lines varied from 1.4 to 1.9 in different experiments, values comparable with those obtained with other viruses and with nucleic acid preparations from tobacco mosaic virus (Bawden & Kleczkowski, 1955, 1959*b*).

Table 5. *Effect of ultraviolet irradiation on the infectivity of a nucleic acid preparation*

Two ml. nucleic acid preparation were exposed to ultraviolet radiation for various times at 1450 $\mu\text{W}/\text{cm}^2$ in a Petri dish of 5 cm. diameter.

'Dark' and 'light' indicate that the French bean test plants were kept after inoculation in darkness and daylight respectively.

Dose of ultraviolet radiation (sec.)	Dilution of nucleic acid preparation	Total number of lesions on eight half-leaves	
		Light	Dark
0	1/20	69	42
0	1/80	12	10
20	1/2	77	21
30	1/2	30	6

Preparations from short virus particles

Harrison & Nixon (1959) found that the lengths of particles of tobacco rattle virus fell predominantly into two categories, 73–77 and 179–192 $\text{m}\mu$, and showed that only the long ones were infective. Both types of particle appeared to have the same gross chemical composition, and no differences were found between their proteins by serological methods. The phenol method offers a way of investigating the possibility that the essential difference between them lies in their nucleic acid components. Preparations of short particles were obtained from purified virus suspensions by rate zonal centrifugation in a sucrose density-gradient. When these were treated with phenol, the resulting preparations had only a trace of infectivity, corresponding exactly to the nucleic acid contributed by the few long particles contaminating the starting material. Evidently the short particles contain non-infective

nucleic acid. Preparations made by phenol treatment of short particles appeared similar in the electron microscope to nucleic acid preparations from unfractionated samples of tobacco rattle virus.

DISCUSSION

By analogy with tobacco mosaic virus, nucleic acid can be expected to be a major constituent of the preparations obtained by treating tobacco rattle virus with phenol. Ultraviolet absorption measurements on the preparations are also consistent with this idea, and the sensitivity of infectivity to inactivation by ribonuclease suggests that the nucleic acid is of the ribose type, like that in all other plant viruses yet examined. Phosphorus analyses too, made by Dr W. S. Pierpoint, suggest that the nucleic acid preparations do in fact contain all, or nearly all, of the virus nucleic acid. Electron microscopy shows that the preparations contain little or no protein in the form of virus rods, and although small fragments of virus protein may well be present, ultraviolet absorption measurements suggest that the amount of protein contamination is small. The infective particles in nucleic acid preparations behave much like those from other viruses. They are inactivated by minute amounts of ribonuclease or by storage *in vitro* at 20°, and few of them are sedimented by high-speed centrifugation for periods in which over 95 % of normal tobacco rattle virus particles sediment. The infective particles may well be delicate thread-like structures of the type seen in the electron micrographs. Like those from tobacco mosaic virus, ultraviolet-irradiated nucleic acid preparations from tobacco rattle virus show the phenomenon of photoreactivation, although neither of the parent viruses does. Presumably the protein and nucleic acid are combined similarly in the two viruses, in a manner that prevents reversible changes occurring in their nucleic acids when the viruses are exposed to ultraviolet radiation. This type of combination may be common to viruses with straight rod-shaped particles.

Although infective nucleic acid preparations can readily be made from a wide range of ribose nucleic acid-containing viruses, such preparations usually appear much less infective than their parent virus suspensions. One possible reason is that an infective nucleic acid particle has a smaller chance than an infective nucleoprotein particle of multiplying when inoculated to a susceptible host. Indirect evidence of this is given by tobacco mosaic virus, nucleic acid preparations from which may increase thirty-fold in infectivity when mixed with virus protein in conditions favouring the formation of rod-shaped particles (Fraenkel-Conrat, 1957). A second reason is that the methods used for extracting the nucleic acid from virus particles may give small yields of high molecular weight material, as for example with potato virus X (Bawden & Kleczkowski, 1959*a*), or may result in damage to some initially infective nucleic acid particles. Furthermore, methods of making infective nucleic acid preparations take no account of the possibility that something in addition to nucleic acid may be needed for infectivity. There are, therefore, many possible explanations for the observation that the fraction of

the infectivity of whole virus which remains in nucleic acid preparations is greater with tobacco rattle virus than other viruses. It has already been pointed out that French bean plants, which were used in work with tobacco rattle virus, may be unusually susceptible to infection by nucleic acid inocula. That virus protein can influence the chance of an infection succeeding is shown by experiments with two strains of tobacco mosaic virus, which caused different numbers of lesions per unit weight when compared as nucleoproteins, but similar numbers when compared as separated nucleic acids (Fraenkel-Conrat & Singer, 1957). It would be interesting to know whether infective units of nucleic acid from one virus have the same chance of multiplying as those from other unrelated viruses when tested in the same host species and conditions. Apparent differences in numbers of nucleoprotein particles inoculated for each infection obtained when different viruses are tested in this way may come from differences in the behaviour of their protein portions during the processes of infection. Nucleoprotein particles of tobacco rattle virus have an unusually low specific infectivity (Harrison & Nixon, 1959), but nucleic acid units derived from them appear to have a similar infectivity to those from other plant viruses.

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