Labelling of Tobacco Mosaic Virus With $^{125}$I

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SUMMARY

A simple and rapid in vitro labelling technique is described for preparing tobacco mosaic virus labelled with $^{125}$I. The technique is versatile and has been used to obtain labelled virus of both high and low specific activity. High specific activity virus has been used in a pilot radioautographic experiment at the electron microscope level, and micrographs were obtained showing virus particles in close association with silver grains. At specific activities of 2 to 3 mc/mg, a rapid loss of infectivity was observed after labelling; this effect was not observed when the specific activity was 1 mc/mg. The sensitivity of detection for high specific activity preparations by scintillation counting and by electron microscope radioautography is discussed and the potentiality of $^{125}$I-labelled virus of both high and low specific activity is indicated.

INTRODUCTION

The observation that isolated tomato fruit protoplasts take up tobacco mosaic virus (TMV) from ambient media (Cocking, 1966) has stimulated an investigation of virus infection in these protoplasts and also of the uptake process itself. Cairns (1962) has discussed the applicability of radioactively labelled virus to infection studies, and the electron microscope radioautographic study by Dales (1963) of vaccinia virus infection is an example of the potentiality of this approach. It was therefore decided to investigate the possibility of preparing radioactive TMV of a sufficiently high specific activity to enable sensitive detection using electron microscope radioautography. Previous radioautographic work with labelled TMV has been at the light microscope level using $^{14}$C-labelled virus (Herridge & Schlegel, 1962; Brants, 1963, 1965). Virus was labelled in these studies by supplying $[^{14}\text{C}]{\text{O}}_2$ to host tissue during virus synthesis. The specific activities obtained using this technique were relatively low although the activity reported by Herridge & Schlegel (1962) represents approximately a 1 to 2% substitution of virus carbon by $^{14}$C. A further disadvantage of this labelling technique is that the virus must be purified from contaminating $^{14}$C-labelled plant material before use.

Anson & Stanley (1941) reported a reaction between iodine and TMV in vitro resulting in the formation of a stable infective virus preparation with iodine bound to the sulphhydryl groups of the protein of the virus. Fraenkel-Conrat (1955) also studied the reaction using $^{131}$I and suggested that stable sulphenyl iodide groups were formed ($-\text{SH}+\text{I}_2\rightarrow-\text{SI}+\text{HI}$). The preferred isotope for radioautography is $^{125}$I (Appelgren, Soremark & Ullberg, 1963) since it has a relatively short half-life (60 days), allowing substantial decay during exposure, and has a low energy emission
(E<sub>max</sub> = 35 kev) approaching that of tritium (E<sub>max</sub> = 18 kev). 131I is not suitable since the energy of emission is high, resulting in poor radioautographic resolution, and the half-life is 8 days, which would result in substantial decay occurring before exposure could be initiated (cf. Cairns, 1962).

A study was therefore made of the possibility of labelling TMV with 125I at high specific activity using this non-biological iodination reaction. The radioautographic potential of this labelled virus at the electron microscope level has been investigated and the limit of detection of labelled virus by scintillation counting and by electron microscope radioautography is discussed. The advantages of this labelling procedure, for preparing high specific activity virus for radioautography and lower specific activity virus for more general application, are discussed.

**METHODS**

**Iodination reaction.** Iodination was carried out in siliconized glass vials by mixing a volume of concentrated virus suspension with a solution of iodine in neutral aqueous potassium iodide. For labelling, carrier-free [125I]sodium iodide (Radiochemical Centre, Amersham) was added to a dilute solution of iodine and a sample of virus added to commence the reaction. The amount of virus added was calculated to be approximately equivalent to the iodine in solution on the basis of each sulphydryl group on each protein subunit of the virus reacting with 2 atoms of iodine, one of which becomes bound (Fraenkel-Conrat, 1955). Table 1 shows example reaction mixtures, one for preparing very high specific activity virus in which the volume is largely determined by the radioactive concentration of 125I and the amount of virus labelled is low, and another for preparing lower specific activity virus in which the volume may be reduced by using more concentrated reagents, and in which the amount of virus labelled is much increased. The mixture was incubated at room temperature for 30 to 40 min. and a slight excess of 0.005 N-sodium thiosulphate added to convert any unreacted iodine to iodide. In the case of high-activity preparations virus was separated from the bulk of the contaminating radioactive iodide by centrifugation in a Spinco SW 39 L rotor at 35,000 rev./min. When the level of activity was lower gel filtration was used. A few crystals of solid sucrose were added to the reaction mixture and the complete mixture layered on to a 4 % agarose column. A short narrow column (1 cm. x 8 cm.) was used since the total sample volume was low. Virus was eluted with the void volume, the iodide being retarded by the gel.

**Radioactivity determination.** Radioactivity was determined by liquid scintillation counting using a Panax scintillation counter with an automatic scaler-timer. Optimum amplifier and H.T. settings for counting 125I were found to be 1100 V with a discriminator bias of 6 V. Samples of 0.01 ml. were counted in 5 ml. of scintillation mixture comprising 2,5-diphenyloxazole (0.4 %) and 1,4-bis-(5-phenyloxazol-2-yl) benzene (0.01 %) in scintillation-grade toluene (70 %) and filtered, spectroscopically pure ethanol (30 %) (Hall & Cocking, 1965). The counting efficiency under these conditions was approximately 15 %.

**Assay of infectivity.** Virus preparations were inoculated on the top 4 or 5 leaves of 2- to 3-month-old *Nicotiana glutinosa* by rubbing with a glass rod. Carborundum powder (500 mesh) was dusted on to the leaves before inoculation. To follow any loss of infectivity in labelled preparations 0.01 ml. samples of dilutions of radio-iodinated
and non-iodinated virus of equal concentrations (0.003 % and 0.0015 %) were inoculated at given intervals on opposite half-leaves of *N. glutinosa* and the ratio of lesion counts appearing after 2 days, between labelled and non-labelled preparations, recorded for each leaf. Two plants were used at each time interval and the results are expressed as the mean for all the leaves used.

**Incubation and preparation of protoplast material for electron microscopy.** One-twentieth ml. of a concentrated suspension of washed protoplasts (Gregory & Cocking, 1965) in 20 % (w/v) sucrose was added to 0.05 ml. of 0.08 % $^{125}$I-labelled virus in 20 % sucrose at a specific activity of 2 to 3 mc/mg. After incubation at room temperature for 6 hr, the protoplasts were fixed by adding an equal volume of cold 12 % buffered glutaraldehyde, washed and post-fixed with 2 % buffered osmium tetroxide. After dehydration in graded ethanol the material was stained with 1 % uranyl acetate in absolute ethanol for 1 hr and embedded in a mixture of n-butyl methacrylate and styrene (7/3, v/v) (Mohr & Cocking, 1967). The material was kept at about 4 ° until after uranyl acetate staining. Blocks were sectioned using glass knives to give large sections for radioautography. A particular feature of this embedding medium is the relative ease of sectioning, allowing a large block face to be sectioned. Silver to gold sections were mounted on carbon-coated molybdenum grids (Maunsbach, 1966), stained with lead citrate (Reynolds, 1963) for 30 min. and coated with a thin layer of carbon (Salpeter & Bachmann, 1964).

**Radioautography.** Grids were attached to glass slides using double-sided sticky tape. The centre of the grid was protected by placing the grid across a strip of polythene, allowing only the edges of the grid to stick. This arrangement prevented emulsion reaching the reverse side of the grid (cf. Salpeter & Bachmann, 1965). The slides were dipped into warm diluted emulsion and drained vertically. Both Ilford L4 and Gevaert NUC 307 emulsions were used diluted 1/4 (w/v) with sterile distilled water. After drying the slides were stored in a light-tight box in a desiccator with silica gel, at 4 °, for 4 to 5 weeks. Grids were then detached and developed individually. The

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**Table 1. Examples of reaction mixtures for iodination of TMV**

(a) High specific activity preparation

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMV</td>
<td>0.86 mg.</td>
</tr>
<tr>
<td>$^{125}$I, $1.44 \times 10^{-3}$ mg. (25 mc)</td>
<td>0.265 ml. carrier-free (as supplied)</td>
</tr>
<tr>
<td>$I_n$, $1.7 \times 10^{-3}$ mg.</td>
<td>0.019 ml. of 0.00485 N-KI in 0.0085 N-KI</td>
</tr>
<tr>
<td>Buffer</td>
<td>0.025 ml., 0.2 M-phosphate</td>
</tr>
</tbody>
</table>

Specific activity obtained, 2 to 3 mc/mg.

Iodination efficiency,* about 50 %

(b) Low specific activity preparation

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMV</td>
<td>4.7 mg.</td>
</tr>
<tr>
<td>$^{125}$I, $0.23 \times 10^{-3}$ mg. (0.1 mc)</td>
<td>0.025 ml., carrier-free.</td>
</tr>
<tr>
<td>$I_n$, $63.5 \times 10^{-3}$ mg.</td>
<td>0.05 ml. of 0.01 N-KI in 0.1 N-KI</td>
</tr>
<tr>
<td>Buffer</td>
<td>0.05 ml., 0.2 M-phosphate</td>
</tr>
</tbody>
</table>

Specific activity obtained, 1 mc/mg.

Iodination efficiency,* about 100 %

* Iodination efficiency is calculated as follows (assuming equivalence between iodine and virus): radioactivity supplied = $x$ mc; proportion of iodine to iodide (w/w) = $y$; amount of iodine bound to virus = $\frac{1}{2} \times x \times y$; amount of virus, = $z$ mg.; theoretical specific activity = $(x \times y) / 2z$; iodination efficiency = (observed/theoretical) $\times 100$. 

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grids were immersed in absolute ethanol for 3½ min. (Stevens, 1966) before development in undiluted D 19 B for 4 min. followed by fixation in 15% sodium thiosulphate for 10 min. All reagents were filtered through Millipore membrane filters (0.45 μ average pore diameter) immediately before use. Specimens were examined in an AEI EM 6 B electron microscope operating at 60 kv with an objective aperture of 50 μ. Under these conditions it was found unnecessary to remove the gelatin to enhance contrast.

RESULTS

Iodination reaction

$^{125}$I-labelled virus has been successfully prepared using the reaction conditions described. The specific activity was found to be determined by the proportion of radioactive to non-radioactive iodine atoms in the reaction mixture. The highest specific activity obtained was 2 to 3 mc/mg, using the reaction mixture shown in Table 1 a. By varying the quantity of $^{125}$I added to the mixture shown in Table 1 b preparations in the 1 to 10 μc/mg. range were prepared. Since all the iodine and iodide present in reaction mixture is in equilibrium, $^{125}$I added as iodide becomes partially converted to iodine ($I^- + I_2 \rightleftharpoons I_3^-$).

To minimize the isotope dilution resulting from this equilibrium when preparing high specific activity virus the amount of carrier iodide added was reduced until the iodine-to-iodide ratio was similar to that used by Anson & Stanley (1941) (0.55). Iodination was observed not to be complete under these conditions (Table 1 a). Fraenkel-Conrat (1955) recommended a concentration of iodide of at least x 10 that of iodine, and an experiment was set up to compare these different ratios under conditions of similar dilution. The result shown in Table 1 was confirmed, the iodination efficiency when the iodine/iodide ratio was 0.1 was 100%, but was reduced when the ratio was 0.55. The amount of virus used in each case was calculated to be equivalent to the amount of iodine in solution. This reduced efficiency for the higher ratio was accepted for high specific activity preparations since isotope dilution resulting from using more iodide outweighs the drop in specific activity due to a low efficiency (about 50%).

Infectivity of labelled virus

Infectivity was retained during labelling. A rapid subsequent loss of infectivity was observed when the specific activity of the preparations was high. Fig. 1 illustrates the loss of infectivity for a preparation at 2 to 3 mc/mg. This rate of loss of infectivity is comparable with that reported for a $^{14}$C-labelled preparation by Herridge & Schlegel (1962). They found a 96% drop in infectivity after 5 months storage for virus at about 50 μc/mg. It appears from these data that loss of infectivity is a function of the specific activity and that a single intrinsic radioactive disintegration is usually lethal to the virus. No significant drop in infectivity was observed for an $^{125}$I-labelled preparation at about 1 μc/mg. during storage for 1 month, and it is suggested that infectivity loss is negligible for preparations of such low activity.

Limit of detection

Modern scintillation counting equipment is capable of counting $^{125}$I at a counting efficiency of near 50% (Rhodes, 1965) and with a background count of 10 to 20 counts/min.
(De Wachter & Fiers, 1967). This figure for mean background sets the limit of
detection as 7 to 9 counts/min. (calculated as twice the standard deviation of the
background). A specific activity of 2 mc/mg. is equivalent to approximately $3 \times 10^{-4}$
disintegrations/min./virus particle, which sets the limit of detection by scintillation
counting at approximately 60,000 virus particles.

The sensitivity of detection of labelled virus by radioautography is a function of the
specific activity, the thickness of the section, the sensitivity of the emulsion and the
geometry of the specimen prepared for radioautographic exposure. These last two
factors have been discussed by Bachmann & Salpeter (1965) and may be estimated as

![Graph](image)

Fig. 1. Loss of relative infectivity of $^{125}$I-labelled virus with a specific activity of
2 to 3 mc/mg. during storage at $-20\degree$.

$1/12$ for L 4 emulsion and $1/3$ for the specimen geometry. Thus only about 3% of
radioactive disintegrations are recorded (ignoring self-absorption of low energy
emissions). In any section the number of rod-shaped virus particles sectioned will exceed the number of complete particles present in the section. This effect is accentuated when thin sections of less than the length of a virus rod are used. The mean proportion of virus length included within a thin section to the entire length of the viruses sectioned has been calculated for sections of different thicknesses (Mayo, to be published). For a 0.1 $\mu$ thin section the value is 0.25, and for a 0.14 $\mu$ section the value is 0.3. The specific activity quoted above of 2 mc/mg. represents approximately 32 $^{125}$I atoms present per virus particle. In a 0.1 $\mu$ the average section of a virus will contain 8 of these. During an exposure period of 60 days an average of 4 will decay, of which 3% would be recorded radioautographically. Using electron microscope radioautography of thin sections with an exposure period of 60 days the sensitivity of detection of the high-specific-activity virus preparation described above would be approximately 1 in 10.
Radioautographic detection

Radioautograms have been obtained showing localization of developed silver grains over areas of protoplast cytoplasm. Few grains were found over empty embedding medium or over control material prepared without incubation in radioactive virus. The developed grains may therefore be interpreted as being due to iodinated virus or breakdown products of this virus. Silver grains were found over vesicles in protoplasts (Pl. 1, fig. 1) and also distributed generally over cytoplasm (Pl. 1, figs. 2, 3). Grains were also found to be localized above regions of the plasmalemma (Pl. 2, figs. 4, 5) and in favourable cases a virus particle could be distinguished below certain of these grains (Pl. 2, fig. 5). This localization at the plasmalemma was also evident in material fixed in the presence of radioactive virus and was therefore due to post-fixation adsorption probably caused by the protein-binding capacity of glutaraldehyde as a fixative. This adsorption of virus to the plasmalemma afforded the opportunity for visualizing individual virus particles in association with silver grains (Pl. 3, figs. 6 to 9) since outside the plasmalemma the background contrast of the empty embedding medium is lower than that of cytoplasm or cytoplasmic vesicles. Virus particles could also only be distinguished in near-longitudinal section when the rod shape enhanced the contrast due to staining. Pl. 3, figs. 6 to 9, are representative of several micrographs obtained showing virus particles with silver grains. Because particles could only be identified in particular orientations, these observations were not common; virus particles were detected in association with only a few per cent of grains seen at the plasmalemma.

These micrographs are fairly representative of the degree of resolution expected from electron microscope radioautography using $^{125}$I. Since $^{125}$I is similar to tritium in its emission characteristics (Ada et al. 1966) it may be assumed from data published by Caro (1962) that the average separation between source and silver grain will be between 0 and 0.1 $\mu$, and that it will seldom exceed 0.5 $\mu$. Ilford L 4 gave the more reproducible results although the developed grain size is about twice that of NUC 307 emulsion and therefore tended to obscure more of the underlying virus rods (Pl. 3, fig. 9).

DISCUSSION

The chief reason for developing this labelling technique was the production of high specific activity virus for use in radioautographic experiments at the electron microscope level. Although the principal interest was in high-activity preparations the technique has been found to have several advantages which also recommend its use for preparing labelled virus when only moderate specific activities are required. The technique is both simple and rapid, and can be carried out under precisely controlled conditions. It is also reproducible, and, particularly for low-activity preparations, the specific activity may be reliably predetermined by adjusting the isotope enrichment of the iodination mixture before use. Large amounts of virus may be labelled to moderate levels of activity, although the amount of isotope required to iodinate quantities of virus much above 1 mg. to high specific activities becomes prohibitive. The principal advantage of this technique is that no biological system is involved in the labelling, the reaction being carried out in vitro using a purified virus suspension. This not only greatly increases the rapidity and reproducibility of labelling, it minimizes purification steps subsequent to labelling.
It is suggested that preparations of low specific activity will be of use for studies of virus uptake both in relation to pinocytosis in the protoplast system (Cocking, 1966) and in more general studies of uptake and transport of virus in whole plants (cf. Kontaxis & Schlegel, 1962; Brants, 1963). These preparations may also be of use as inert macromolecular markers for uptake studies on cell systems unrelated to plant virology (cf. Ryser, 1967).

The maximum specific activity produced was 2 to 3 mc/mg., which compares favourably with corresponding 14C-labelled preparations. This level of radio-iodination represents about 3 % of the iodine atoms bound to the virus being radioactive, whilst this specific activity for 14C-labelled virus represents approaching a 100 % substitution of virus carbon by 14C. 125I also has a better radioautographic resolution than 14C at the light microscope level owing to its low energy emission. A rapid loss of infectivity was recorded for this high-activity preparation, which limits its useful life to a few hours after preparation, and it seems likely that this activity is close to the useful limit. It was concluded that for lower-activity preparations infectivity loss occurred at a much slower rate, extending the useful life of preparations in the 1 to 10 μc/mg. range to at least a month.

The technique for which the preparation of high specific activity virus was principally developed was radioautography at the electron microscope level. Virus particles have been found in thin section in clear association with silver grains. The sensitivity of this technique for detecting labelled virus has been discussed and as an approximation it has been concluded that for a preparation at 2 mc/mg. a 2-month exposure would result in the detection of 10 % of the virus present in a thin section. Thus sensitive detection with this technique is only possible when very high-specific-activity virus is used.

Under the conditions of incubation used in this investigation infection was not initiated and no conclusions concerning the fate of virus taken up were reached.

Dales (1963) was able to follow the uptake and development of vaccinia virus by certain animal cells following infection with labelled virus, and it is suggested that a radioautographic study following direct infection of a single cell system with high specific activity 125I-labelled virus could yield fundamental information concerning the fate of an infecting TMV particle.

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REFERENCES


M. A. MAYO AND E. C. COCKING


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EXPLANATION OF PLATES

**PLATE 1**

Fig. 1. Radioautogram showing silver grains over vesicles within the cytoplasm of a protoplast. Emulsion L4.

Fig. 2. An area of cytoplasm with label generally distributed. Emulsion NUC 307.

Fig. 3. Silver grains are seen generally distributed over the cytoplasm. Emulsion NUC 307.

**PLATE 2**

Fig. 4. Radioautogram showing the localization of label at the plasmalemma. Emulsion L4.

Fig. 5. Another region of plasmalemma with silver grains. A virus particle (arrowed) is shown below one of the grains. Emulsion L4.
Plate I

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Plate 3

Fig. 6. An aggregate of two virus particles is shown with a large silver grain at one end. Emulsion L4.

Fig. 7. A virus particle is shown adhering to the plasmalemma with a large silver grain near. Emulsion L4.

Fig. 8. A virus particle is shown in oblique section at one end of the grain. There is a characteristic tramline effect due to selective staining of the virus. Emulsion L4.

Fig. 9. A complete virus particle is shown in near longitudinal section. The silver grain is small. Emulsion NUC 307.