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Inhibition of Multiplication of Tobacco Mosaic Virus in Protoplasts by Antibiotics and its Prevention by Divalent Metals

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SUMMARY

At concentrations that inhibit bacterial growth, some antibiotics including gentamicin completely inhibited virus multiplication in protoplasts, and other antibiotics partially inhibited virus multiplication. The inhibition caused by each antibiotic was largely prevented by adding a divalent metal; $MnCl_2$ was more effective than $CaCl_2$ and other salts of divalent metals when added at 10 mM to the incubation medium. When added immediately after infection, 1 μg/ml of gentamicin halved the final virus concentration and 3 μg/ml completely inhibited virus multiplication, although 10 μg/ml was required to stop bacterial growth. Gentamicin inhibited virus multiplication even when added 24 h after virus inoculation. Also, when protoplasts were exposed to gentamicin for only 1 or 2 h, either immediately after inoculation or 2 h later, the virus concentration was considerably decreased.

Gentamicin seemed not to affect virus multiplication in whole plants. Sap from *Dianthus barbatus* also strongly inhibited virus multiplication in protoplasts but, unlike gentamicin, it acted in the presence of $MnCl_2$. By contrast, chelating agents such as 1 mM-EDTA or 5 mM-potassium citrate were strong inhibitors of virus multiplication that were inactive in the presence of $MnCl_2$. It is suggested that gentamicin and other antibiotics may chelate metals from the protoplast membranes, thus disorganizing their function and affecting virus multiplication.

INTRODUCTION

We recently reported that TMV multiplication was inhibited when $CaCl_2$ was omitted from the medium containing antibiotic in which TMV-inoculated tobacco protoplasts were incubated, and that this inhibition was not prevented when $CaCl_2$ was added to the medium 4 h after virus inoculation. Replacing $CaCl_2$ by $MgCl_2$ partially inhibited TMV multiplication (Kassanis & White, 1974). We suggested that Ca was needed for virus multiplication or indirectly influenced multiplication by preventing derangement of the structure and function of the protoplast membranes and that Mg could partly replace Ca. This paper reports that the inhibition of virus multiplication is caused by the antibiotics added to the incubation medium and that $CaCl_2$ and other salts of divalent metals prevent this inhibition.

METHODS

The methods used were as described by Kassanis & White (1974) except that, for most of this work, we used a simplified incubation medium containing 13·2 % (w/v) mannitol, 10 mM-$MnCl_2$ and either 10 μg/ml gentamicin or 100 μg/ml carbenicillin. The same amounts

Table 1. *Prevention by $CaCl_2$ of the inhibition of virus multiplication caused by gentamicin*

Incubation medium	TMV yield (μg/10^6 protoplasts)
13·2 % mannitol	200
13·2 % mannitol + 10 mM-$CaCl_2$	200
13·2 % mannitol + 10 μg/ml gentamicin	0
13·2 % mannitol + 10 μg/ml gentamicin + 10 mM-$CaCl_2$	226

of virus were produced in protoplasts incubated with either antibiotic, provided $MnCl_2$ was present. To obtain protoplasts from tobacco leaves (*Nicotiana tabacum* L. White Burley type cv. Judy's Pride), we used 0·4 % Macerozyme and 1 % cellulase and washed them in 13·2 % (w/v) mannitol only. The infection medium was as before, containing 1 μg/ml TMV and 2 μg/ml poly-L-ornithine in 10 mM-potassium citrate buffer, pH 5·2. Inoculated protoplasts were incubated for 2 days at 27 °C under lights.

Fluorescent antibody staining was done as before but the percentage fluorescing was calculated from the total number of protoplasts present in the microscope fields counted, because the criterion used to exclude dead cells seems very subjective. We are also concerned that percentage infection does not estimate virus multiplication accurately because the variable intensity of fluorescence suggests that not all protoplasts contain the same amount of virus.

For infectivity tests on plants, inoculations were made without Carborundum, and the TMV used as a standard was newly purified to ensure that its specific activity was high. The amounts of virus estimated by infectivity tests agreed closely with those estimated by electron microscopy, using a known concentration of polystyrene latex particles for comparison (Coutts, Cocking & Kassanis, 1972). Protoplast extracts were always examined in the electron microscope to decide, from the density of virus particles, how much the extracts should be diluted for the infectivity tests. Extracts from control protoplasts were tested either at 1/100 or 1/200 dilution.

RESULTS

Inhibition of virus multiplication by antibiotics

To find how $CaCl_2$ helped the multiplication of TMV it was necessary to simplify the incubation medium. After infection the protoplasts are normally washed 3 times with 13·2 % mannitol solution containing 0·1 mM-$CaCl_2$, but omitting $CaCl_2$ from this solution did not affect the amount of virus produced. We next examined our modification of Aoki & Takebe's (1969) incubation medium (13·2 % mannitol, 0·2 mM-KH_2PO_4, 1 mM-KNO_3, 0·1 mM-$MgSO_4$, 10 mM-$CaCl_2$, 1 mM-KI, 0·01 μm-$CuSO_4$ and gentamicin 10 μg/ml, Kassanis & White, 1974), successively omitting each of the mineral salts except $CaCl_2$ without affecting virus multiplication. Of the basic constituents – mannitol, gentamicin and $CaCl_2$ – mannitol is indispensable to living protoplasts so we tried the four combinations possible with the other two substances. Table 1 shows that it was not, as previously thought, the absence of $CaCl_2$ but the presence of gentamicin that prevented virus multiplication, and that this inhibition was neutralized by $CaCl_2$. In fact, the virus multiplied well in protoplasts kept in mannitol alone.

Having found that $CaCl_2$ prevented the inhibition caused by gentamicin, we tested other metal salts (Table 2). All the salts of bivalent metals we tried tended to prevent inhibition although with some only very inefficiently, but KNO_3 had no effect. In this and several other

Table 2. *Prevention by different salts of the inhibition of virus multiplication caused by gentamicin*

Salt added at 10 mM*	TMV yield (μg/10^6 protoplasts)
$CaCl_2$	72
$MnCl_2$	252
$MgCl_2$	8
$ZnSO_4$	22
$BaCl_2$	100
$FeCl_2$	2
KNO_3	0
None	0

* In addition to these compounds, the incubation medium contained 13·2 % mannitol and 10 μg/ml gentamicin.

Table 3. *Effect of $MnCl_2$ concentration on prevention of the inhibition of virus multiplication caused by gentamicin at 10 μg/ml*

	TMV yield (μg/10^6 protoplasts)		
$MnCl_2$ (mM)	Expt. 1	Expt. 2	Expt. 3
0	0	0	0
0·5	0·2	—	—
1	16	—	14
2	90	—	50
3	112	—	90
4	200	—	—
5	160	100	70
10	—	224	100
20	—	200	—

comparisons, $MnCl_2$ prevented the inhibition of virus multiplication much better than $CaCl_2$. The inhibitory effect of gentamicin was always prevented by 10 mM-$MnCl_2$, sometimes by less (Table 3), perhaps depending on the physiological state of the protoplasts. We standardized our tests by using 10 mM-$MnCl_2$ instead of $CaCl_2$ in all subsequent experiments.

Antibiotics are normally used in protoplast cultures to control the growth of bacteria and fungi, but always in the presence of $CaCl_2$. Aoki & Takebe (1969) found that actinomycin D and chloramphenicol had no effect on virus multiplication when added to the incubation medium in addition to the antibiotics they used to control bacteria and fungi. More recently, however, Motoyoshi, Watts & Bancroft (1974) found that amphotericin B, tetracycline and kanamycin somewhat inhibited virus multiplication even in the presence of $CaCl_2$. Carbenicillin and gentamicin, at the concentrations used in this work, are active against bacteria but still permit normal protoplast metabolism in the presence of $CaCl_2$ (Watts & King, 1973). It was of interest, therefore, to test whether various other antibiotics were inhibitory in the absence of a bivalent metal. Table 4 shows that all these antibiotics reduced the amount of virus produced, some to nothing.

The antibiotics inhibited bacterial growth at the concentrations used, both with $MnCl_2$ or without, and protoplasts appeared normal except that in tetracycline and chlortetracycline they became brown unless $MnCl_2$ was added. The antibiotics we found to have least effect on TMV multiplication were carbenicillin, tetracycline and actinomycin D. Without

Table 4. *Inhibition of virus multiplication by different antibiotics*

Antibiotic	TMV yield (μg/10^6 protoplasts)	
	Without $MnCl_2$	With $MnCl_2$ (10 mM)
Gentamicin 10 μg/ml	0	105
Carbenicillin 100 μg/ml	80*	120*
Nystatin 25 units/ml	24	60
Chloramphenicol 100 μg/ml	1	37
Streptomycin 30 μg/ml	0	95
Kanamycin 50 μg/ml	0	37
Chlortetracycline 30 μg/ml	10*	160*
Tetracycline 30 μg/ml	143*	254*
Actinomycin D 10 μg/ml	100	224

* Average virus yield from 3 experiments.

Table 5. *Effect of carbenicillin and $MnCl_2$ on percentage infection and virus concentration in protoplasts*

Incubation medium*	% infection	Serological titre	TMV yield (μg 10^6 protoplasts)	
			Electron microscopy assay	Infectivity assay
Carbenicillin 100 μg/ml	32	1/8	30	56
Carbenicillin 100 μg/ml + 10 mM-$MnCl_2$	54	1/16	54	142
10 mM-$MnCl_2$	56	1/16	68	142

* The incubation medium also contained 13·2 % mannitol.

Table 6. *Increase of virus concentration in protoplasts and control of yeasts by $MnCl_2$*

Incubation medium	TMV yield (μg/10^6 protoplasts)	Growth of yeasts
13·2 % mannitol	60	+
13·2 % mannitol + 100 μg/ml carbenicillin	26	+
13·2 % mannitol + 100 μg/ml carbenicillin + 25 units/ml nystatin	42	—
13·2 % mannitol + 100 μg/ml carbenicillin + 10 mM-$MnCl_2$	105	—

$MnCl_2$, tetracycline affected the appearance of the protoplasts, while the inhibitory effect of actinomycin D on DNA-dependent RNA synthesis is well known. Carbenicillin therefore seemed to be the most useful of the antibiotics we tried, and in one experiment we studied its effect on the percentage of protoplasts infected and the virus yield – estimated serologically, by infectivity test and by electron microscopy. Table 5 shows that carbenicillin alone decreased percentage infection of protoplasts as well as total virus produced but that both effects were prevented by adding $MnCl_2$. In another experiment, fluorescent antibody staining did not detect any infection of protoplasts incubated with gentamicin and mannitol only.

$MnCl_2$ not only prevents the inhibition of virus multiplication but completely stops yeast growth, as none was seen under the microscope. However, the control of yeasts is not the reason for the higher virus yield because they can also be controlled by adding the fungicide

nystatin to the medium, and nystatin does not increase virus concentration (Table 6). Also, when nystatin was used with gentamicin there was complete control of yeasts but no virus was produced unless $MnCl_2$ was also added.

A possible mechanism of the inhibition

Gentamicin is produced by the actinomycete *Micromonospora purpurea*. Extracts from some micro-organisms and flowering plants strongly inhibit infection when inoculated to plants together with virus. That gentamicin has no effect on TMV multiplication in plants was shown by injecting it at 10 μg/ml into the intracellular spaces on one half of the lamina of tobacco leaves cv. Xanthi-nc, while the other half was injected with water, and inoculating the whole leaf with 1 μg/ml of TMV 1, 2 or 3 days later. The number and size of lesions were the same in both half leaves. Also, when gentamicin was added to the inoculum, there was no decrease in the number of lesions produced. By contrast, as little as 3 μg gentamicin/ml completely inhibited TMV multiplication in protoplasts. In one experiment, 1 μg/ml of gentamicin halved the virus concentration and no virus was produced with 3 μg/ml when the antibiotic was used alone, while 200 μg TMV/10^6 protoplasts was produced using 10 μg/ml of gentamicin together with 10 mM-$MnCl_2$.

Most virus inhibitors influence infection rather than virus multiplication, and so do not act when introduced 3 to 4 h after virus inoculation. In protoplasts, new TMV is detectable between 6 and 8 h after inoculation (Kassanis & White, 1974). However, when 10 μg/ml gentamicin (without $MnCl_2$) was added 0, 2, 3, 4 and 6 h after inoculation, no virus was produced by 48 h after inoculation, whereas the control (gentamicin and 10 mM-$MnCl_2$) produced 200 μg TMV/10^6 protoplasts. When addition of gentamicin was delayed for 24 h after infection 60 μg/10^6 protoplasts was produced as against 105 μg for the control. Therefore, multiplication can be inhibited at any time after inoculation. In fact inhibition was considerable when gentamicin was present in the incubation medium for 1 h only; when added immediately after inoculation and 1 h later removed by centrifuging the protoplasts, washing them and resuspending in medium without gentamicin, the virus yield was decreased to 12 μg/10^6 protoplasts as against 164 μg for the control. Again, little virus was obtained when the protoplasts were exposed to gentamicin from 4 to 6 h after inoculation. The virus yield of such protoplasts was 6 μg/10^6 protoplasts and of the control, 94 μg.

In trying to understand the mechanism of inhibition in protoplasts we tested the sap of sweet william plants (*Dianthus barbatus* L.) which is a strong inhibitor of virus infection in plants. When clarified sweet william sap was mixed at dilutions 1/10 and 1/50 with TMV and inoculated to tobacco Xanthi-nc, no lesions developed at dilution 1/10, and their number was decreased by 90 % at dilution 1/50. Similarly, virus multiplication was much decreased when sweet william sap was added to protoplasts, but unlike gentamicin, the effect was not reversed by adding $MnCl_2$. In one experiment, clarified sweet william sap (final dilution 1/100) was added to the incubation medium with 10 mM-$MnCl_2$ and 100 μg/ml carbenicillin 0, 2, 3, 4 and 6 h after inoculation. For control, an equal amount of clarified tobacco sap was added in the incubation medium. The amount of virus produced by 10^6 protoplasts was 0, 0, 0, 16 and 20 μg respectively for the different time intervals and 224 for the control exposed to tobacco sap. All the protoplasts looked normal. The inhibitory effect of gentamicin, therefore, differs from that caused by sap extracted from sweet william plants.

Gentamicin might inhibit virus multiplication by chelating metals and disorganizing the protoplast membranes. If so, a similar inhibition of virus multiplication should take place when other chelating agents are added to the incubation medium. An experiment was made in which 1 mM-EDTA either with or without 10 mM-$MnCl_2$ was added to the antibiotic-free

incubation medium and the medium adjusted to pH 5·4. No virus was produced without $MnCl_2$ and 106 μg/10^6 protoplasts with $MnCl_2$. In a similar experiment, 5 mM-potassium citrate (pH 5·4) was used with and without 10 mM-$MnCl_2$. No virus was produced without $MnCl_2$ and 398 μg virus/10^6 protoplasts with $MnCl_2$. The protoplasts appeared normal after 48 h incubation in EDTA while they died in potassium citrate alone, but appeared normal where $MnCl_2$ was also added. It is possible, therefore, that gentamicin acts like EDTA and potassium citrate in chelating metals.

To test whether gentamicin binds $MnCl_2$, a mixture of antibiotic and $MnCl_2$ was run down a Sephadex column at the ratio of 10 μg antibiotic/ml to 3 mM of $MnCl_2$, a concentration which completely reversed the inhibition by gentamicin (Table 3). The column of Sephadex G-10 (1·1 × 9·3 cm) was equilibrated in water, and the effluent was monitored using a Uvicord detector. Gentamicin emerged as a small peak, or with one sample as two small peaks, a little after the void volume (37 ml) of the column. $MnCl_2$, which was located by testing samples of the effluent with NaOH, emerged 5 to 6 ml later, well before the bed volume of the column; although it was spread out, it was satisfactorily separated from gentamicin. The pooled fractions containing gentamicin were tested for inhibition of infection on protoplasts, alone and after adding 10 mM-$MnCl_2$. Alone, the fractions were inhibitory but not after adding extra $MnCl_2$. The results suggest that either gentamicin does not bind $MnCl_2$ or any complex formed breaks down during chromatography.

DISCUSSION

Judging by the percentage of infected protoplasts and the yield of virus per protoplast, the overnight method of producing protoplasts, used in this work, is as good as the two-step method of Aoki & Takebe (1969). In fact, we believe that our method is more gentle, and thus the effects we found with the different antibiotics are not caused by any abnormality in the protoplasts inherent to the method.

Gentamicin and other antibiotics, at concentrations that stopped bacterial growth, inhibited virus multiplication in protoplasts, but this was prevented by adding 10 mM-$MnCl_2$ to the incubation medium. It is well known that 'virus inhibitors', such as extracts from microorganisms or some flowering plants, act in the first 3 h after inoculation by preventing the initiation of infection in plants. Gentamicin was active even 24 h after inoculating protoplasts and did not inhibit infection in plants. That the action of gentamicin was dissimilar to that of most virus inhibitors was shown by adding sap of sweet william leaves to the medium. This sap strongly inhibits infection of tobacco plants. Although it also strongly inhibited virus multiplication in protoplasts, the effect was not reversed by adding $MnCl_2$. By contrast, inhibition by EDTA or potassium citrate was reversed by adding $MnCl_2$, and this suggests that gentamicin and other antibiotics act as chelating agents. Exposing the protoplasts to gentamicin for only 1 h was sufficient to inhibit virus multiplication, so it is possible that it chelates metals from the membranes, disorganizing their function and thus affecting virus multiplication. Although potassium citrate is present in the infection medium, the protoplasts are not affected, probably because they are exposed for only 10 min in this medium. Considering the harmful effect of potassium citrate it might be advisable to replace it with another buffer.

It is also of some interest that the 'virus inhibitor' from sweet william acted on virus multiplication in protoplasts differently than in plants. In plants the effect is usually on the initiation of infection, but in protoplasts the substance had considerable inhibitory effect even 6 h after inoculation, when it is known that new virus has been formed. The reason for the

difference might be in the method of application. In plants the inhibitors are either rubbed together with the virus on to the leaf surface or they are rubbed on to the leaf surface at different times after inoculation. This method probably does not allow sufficient inhibitor to enter the cells to inhibit virus replication. By contrast, adding the inhibitor in the medium probably allows more to enter the protoplasts, possibly by pinocytosis. If this is true, then the hypothesis that inhibitors only compete with virus for receptor sites at the beginning of the infection process (Van Kammen, Noordam & Thung, 1961) is incorrect.

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