PLANT VIRUS SYNTHESIS AND THE ABNORMAL PROTEIN CONSTITUENTS OF INFECTED LEAVES

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INTRODUCTION

In the last few years, several groups of research workers[1,2,3,4,5,6] have drawn attention to the existence, in plants infected with viruses, of protein constituents that have the same immunological specificity as the virus, but are non-infectious and lack ribonucleic acid. The role of these constituents is not yet known. We have shown, in two recent preliminary notes[7,8], that labelled amino acids were incorporated more rapidly into these constituents than into the infectious particles. In view of this fact, it was possible to eliminate the hypothesis that they were produced from the infectious particles by degradation and loss of ribonucleic acid.

Since then we have continued this line of research, and now consider that we can provide clear evidence in favour of the idea that, at least in the case of tobacco mosaic virus, these abnormal protein constituents are the precursors of the protein part of the infectious particles.

In the current literature no general term has been accepted to designate collectively the abnormal constituents which accompany tobacco mosaic virus in an infected plant. In accordance with our previous suggestion[4,5] we shall call them "soluble antigens". This term has for a long time been applied to those constituents, which are perhaps analogous, present in animal tissues infected by vaccinia virus.

METHODS

1. *Culture of the virus*

Leaves of *Nicotiana tabacum* var. White Burley are infected by means of a 0.5 % solution of tobacco mosaic virus (stock provided by Dr. BAWDEN) and cut up in fragments about 10 cm square. The fragments are divided into batches which are physiologically equivalent, that is, which contain equal numbers of fragments from the different plants used, and from leaves of different ages. They are exposed to light in a moist chamber for 24 hours, washed in water to eliminate, as far as possible the excess of virus that has remained on the surface, and then laid out, lower side towards the light, on a thin layer of cotton wool soaked in half-strength Vickery medium. This is spread out on the bottom of closed glass dishes, 1.5 cm high and with a surface area of one square metre. These dishes are illuminated by a grid of fluorescent tubes. The temperature in the dishes varies between 23°C and 26°C. Each experimental sample consists of one or several entire batches of fragments.

2. *Incorporation of* $^{14}CO_2$

At the moment chosen for estimating the rate of incorporation, 400 μc of $^{14}CO_3Ba$ are placed in a small glass vessel in each dish. When the dish has been closed again, lactic acid is added, and the released $^{14}CO_2$ is at once uniformly distributed by alternately compressing two rubber bladders attached at opposite corners of the dish.

The incorporation of CO_2 can be interrupted at a known time, since the dishes can be opened and the leaves taken out very rapidly. The collected leaves are immediately crushed with an homogenizer and frozen. Incorporation of CO_2 over a relatively short time (30 min) can thus be studied under conditions that are sufficiently reproducible.

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It was essential, if the results were to be interpretable, that the specific acitivty of the free amino acids should remain stable or increase regularly during incorporation of the CO_2. In previous experiments, carried out in conditions analogous to those described above, one of us[9] followed changes in the specific radioactivity of CO_2 liberated by means of ninhydrin from the total free amino acids, and the CO_2 released from free glutamic acid by a specific decarboxylase. It was shown that the specific radioactivity of liberated carbon dioxide increased for 2 to 4 hours after adding $^{14}CO_2$, and then diminished. Incorporation in these experiments was therefore allowed for only 30, 60, or 120 minutes.

3. *Isolation of the virus and the labelled soluble antigens, and measurement of their specific radioactivity.*

The techniques used are based upon data derived from a previous investigation[5]. The virus is separated from the soluble antigens by centrifugation of the leaf extracts at 30,000 r.p.m. for 1 hour (rotor No. 30 of the Spinco quantitative centrifuge).

The method of COMMONER, modified as described[5], was used for purification of the virus present in the sediment. The soluble antigens in the supernate are precipitated by adding an excess of antiserum, which is obtained from a rabbit previously injected with purified virus. The precipitates are washed with 0.9 % NaCl, and then both soluble antigens and purified virus are treated with 10 % trichloroacetic acid, alcohol and ether. The dry powders so obtained are finely ground and their radioactivity is measured by means of a counter with mica window. Geometric conditions are constant, and sufficiently thin layers are used to obviate the need for a correction for self absorption.

In the case of the virus, the radioactivities so measured are related to unit weight of the powder. In order to determine the specific activity of soluble antigens, however, we need to know the proportions of antigen and antibody present in the immunological precipitate, and this proportion varies according to the relative concentrations of antigen and antibody in the solution in which precipitation is produced.

4. *Determination of the proportion of antigens and antibodies in the immunological precipitates of soluble antigens*

A large quantity of labelled soluble antigens is prepared by bringing into contact with $^{14}CO_2$ leaves of old plants that have been infected while very young and contain amounts of soluble antigens up to 20 % of the weight of the virus.

Soluble antigens are isolated from 400 g of leaves so treated, by alternate precipitations at pH 4.7 and pH 3.8 of extracts previously freed of virus by rapid centrifugation[5]. These precipitations are repeated until the specific radioactivity of the product is constant.

Electrophoretic tests on this material had previously shown[5] that it contained no appreciable quantity of the normal plant proteins. If there were such a contamination however, it would have little importance for the present work. In fact the specific radioactivity of the normal proteins is very similar to that of the soluble antigens when there is no growth of the virus during incorporation. This is the case in these experiments.

Equal volumes of antigen solutions of various concentrations are then added to a constant volume of antiserum. The specific radioactivity of the immunological precipitates is a measure of the proportion of antigens and antibodies that they contain. A graph expressing the relationship between the antigen content of the immunological precipitates and their weight can in this way be constructed. We used such graphs to determine the soluble antigen content of the precipitates that we had to study, and of which we knew only the dry weight. In all these determinations we took the indispensable precaution of reproducing the same conditions in all cases, and especially of obtaining a constant concentration of antibody in the mixture from which the precipitate is formed.

5. *The importance of possible contamination of immunological precipitates of soluble antigens by material of high specific radioactivity*

The soluble antigens represent only 8–16 % of the total weight of the immunological precipitates obtained in our experiments. An important source of error in the determination of their specific radioactivity could result, therefore, from the adsorption on the precipitates of material of high specific radioactivity and low molecular weight from the extracts. This possibility had to be investigated with care. For this purpose non-infected leaves are supplied with $^{14}CO_2$ and extracts are prepared from them under the usual conditions. A solution of non-labelled soluble antigens and antiserum is added to these extracts, and the immunological precipitate formed is washed in the usual way. The radioactivity present, which is hardly detectable, is equal to 2 % of the radioactivity of the normal proteins of the extract.

Since the immunological precipitates of soluble antigens with which this work is concerned have specific radioactivity at least four times as great as that of the normal proteins, we can estimate that the maximum error in the measurement of specific radioactivity of soluble antigens that

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could result from contamination would be 0.5 %. This technique is therefore remarkably successful in eliminating a source of error common in research on the incorporation of isotopes into proteins.

6. *Measurement of the rate of growth of the virus during incorporation of* $^{14}CO_2$

During growth of the virus, repeated measurements of the quantity present are made at short intervals before and after the period of $^{14}CO_2$ incorporation. The rate of growth of the virus during the short period of incorporation can be determined with sufficient accuracy from the curve so obtained. At each determination a sample of leaf fragments of known weight is taken. The fragments are homogenized and the extract is frozen at —20°C. After it has been thawed, the extract is centrifuged for 10 min at 30,000 r.p.m., and an excess of antiserum is added to the supernate. The precipitates are washed as before, and after mineralisation, their nitrogen content is measured.

Parallel precipitations of known quantities of purified virus are carried out under the same conditions, and a graph is made expressing virus content as a function of the weight of immunological precipitate. From this graph the quantity of virus present in the precipitates used to establish the growth curve can be calculated.

These precipitates, it should be noticed, contain both virus and soluble antigens. However, at the stage of virus growth used in these experiments there are only very small amounts of soluble antigens (less than 1 % of the amount of the virus) and their presence in the immunological precipitates is not a source of error that need be taken into account.

RESULTS

Even a superficial examination of the results obtained by the methods described is significant. Let us consider Fig. 1, which shows the changes in the specific radioactivities of the soluble antigens, the virus and the normal proteins (fraction remaining in solution after centrifugation for 1 hour at 90,000 *g*) in leaves that have been brought into contact with $^{14}CO_2$ three days after infection when the virus is actively growing.

Previous experiments[9] showed that under the conditions used there is no increase in the normal proteins. The increase in their specific radioactivity probably represents, therefore, the slow renewal of their amino acids by exchanges between these and the free amino acids, which have a high specific radioactivity. There is also an analogous renewal of the virus particles when no growth is taking place, but this is much slower than that of the normal proteins. The fact that in the present experiment the specific radioactivity of the virus increases much more rapidly than that of the normal proteins is easily explained by the incorporation of amino acids into the virus during its rapid increase in quantity. The observed development of radioactivity in the normal proteins and virus is therefore what might have been expected. On the other hand, increase of the specific radioactivity of the soluble antigens is at first sight very surprising. After only 30 minutes it has reached a value which is 67 times greater than that of the normal proteins, and 180 times greater than that of the virus.

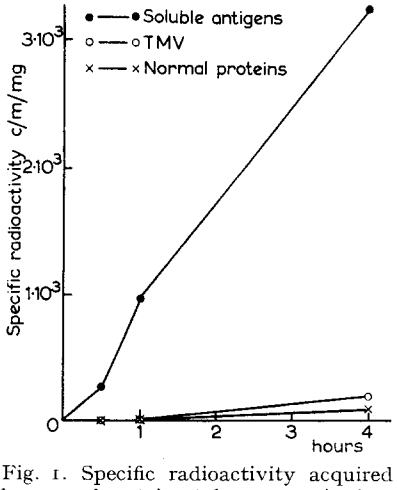

Fig. 1. Specific radioactivity acquired by normal proteins, tobacco mosaic virus and its soluble antigens, in infected tobacco leaves, illuminated in the presence of $^{14}CO_2$.

This considerable incorporation of free amino acids could in principle be explained by a very rapid increase in the quantity of soluble antigens. However, the increase in the soluble antigens, far from being 180 times as rapid as that of the virus, is negligible

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during the period of incorporation to be considered. We are thus led to explain the high rate of incorporation of free amino acids into the soluble antigens either by imagining an abnormally high interchange between the free amino acids and those of which the soluble antigens are formed, or by the hypothesis that the soluble antigens are synthesised with great rapidity and used at the same rate in the synthesis of the virus, of which they might be the direct precursors.

The first of these hypotheses is unlikely, since such rates of renewal by exchange between protein and free amino acids have never been reported, even in the case of rapidly growing bacteria, where the rate is very low, perhaps even reaching zero[10]. The value of the second hypothesis, which is much more probable, has been tested quantitatively by experiments designed for this purpose. The principle of these experiments is as follows. The rate of increase of the quantity of virus and the specific radioactivity of soluble antigens are determined. In accordance with the hypothesis that the soluble antigens are the direct precursors of the virus, we calculate the specific radioactivity that should be acquired by the virus at a certain time after the introduction of $^{14}CO_2$. If the hypothesis is correct, the calculated radioactivity will be identical with the measured radioactivity of the virus.

In order to make these calculations, we assumed that the amount of soluble antigens incorporated into the virus is equal to the increase in amount of virus, and that the specific radioactivity of the incorporated antigens is equal to the specific radioactivity of the antigens that we isolate.

An example of calculation of this type is given in Table I, in which the calculated increase in radioactivity of the virus each hour is equal to the product of the increase in the quantity of virus during this hour and the mean of the specific radioactivities of the soluble antigens at the beginning and at the end of the same hours.

TABLE I

Time (hours after infection)	*Virus present (mg virus/g leaves)*	*Virus synthesized in 1 hour (mg virus/g leaves)*	*Specific radioactivity of soluble antigens (c/min/mg dry wt)*		*Radioactivity introduced into virus in 1 hour*	*Specific radioactivity of virus (c/min/mg dry wt)*	
			measured	*mean*		*calculated*	*measured*
95	1.590		0			0	0
		0.055		1015	55·7		
96	1.645		2030			34	32
		0.055		3865	213		
97	1.700		5700			158	190

It should be noticed that the correction that might have been introduced into the calculations on account of the ribonucleic acid in the virus is negliglible, since ribonucleic acid represents only 6% of the total, and has a radioactivity close to that of the proteins. This correction was therefore omitted.

It can be seen that the calculated and measured values for the radioactivity of the virus are in excellent agreement, if we take into account the type of experiment. The hypothesis that the soluble antigens are the precursors of the protein part of the virus therefore accounts perfectly for the experimental facts.

How then can we picture the behaviour of the soluble antigens inside a cell which is synthesising virus? At the end of the 95th hour we found, for each gram of leaf, 13 γ of soluble antigens and 1645 γ of virus. The soluble antigens, therefore, represent 0.8% of the quantity of virus. Since the amount of virus synthesized in one hour is

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approximately equal to 3.4% of the amount present, the total amount of soluble antigens synthesised in an hour is four times as great as the amount existing at a given moment. The replacement of the antigen molecules that are incorporated into the virus particles must therefore take place extremely rapidly, and we can predict that the soluble antigens should have a specific radioactivity very close to that of the free amino acids, during the whole period of our experiments.

These indirect conclusions fit in perfectly with our observations on the development of the specific radioactivity of the normal proteins. If we suppose that the mean specific radioactivity of the free amino acids is close to that of the soluble antigens, we come to the conclusion that the renewal of the amino acids of the proteins is of the order of 2% per hour, which is the value previously arrived at by the study of the changes in the specific radioactivity of free glutamic acid and the glutamic acid of proteins in detached tobacco leaves[9].

Finally, we have compared the rate of incorporation of $^{14}CO_2$ into soluble antigen and virus of old plants, where there is no longer an increase of these constituents. This rate of incorporation, corresponding this time to exchanges between the free amino acids and the amino acids incorporated into proteins, was very low, and was approximately the same in the virus and in the soluble antigens. This is an additional argument in favour of the idea that, in a leaf where the virus is growing, the enormous radioactivity acquired by the soluble antigens can only be explained by their rapid synthesis.

DISCUSSION AND CONCLUSIONS

Before comparing the preceding results with those mentioned in the literature, it is necessary to make two preliminary observations. During previous research[5] we have only been able to detect soluble antigens in plants that have been developing systemic infections for several months. Contrary to what we thought at that time, these antigens exist from the beginning of a direct infection, but they are 20 times less abundant, for a given weight of virus, than in the case of systemic infections of long standing, and these small quantities escaped us. Our present results are in agreement with those obtained in other laboratories[3,6].

We must point out further that the fraction that we call soluble antigens contains a group of abnormal constituents that are serologically similar to the virus, but lack ribonucleic acid, are non-infectious, and have a lower sedimentation rate. We showed previously[5] that there were at least two of these constituents. One of them at least shows all the characteristics of the constituents X of TAKAHASHI *et al.*[2,3]. The material on which we have worked is on the other hand identical, in whole or in part, to the abnormal constituents studied by COMMONER *et al.*[6].

The relations that might exist between the soluble antigens and the virus have already been studied by DELWICHE *et al.*[11] with the help of ammonium labelled with ^{15}N. The incorporations observed by these authors in the soluble antigens are only two or three times higher than those in the virus. This difference between their results and ours can be explained by the long duration of their experiments (24 hours instead of 1 or 2 hours); the incorporation in the virus rapidly approaches the incorporation into the soluble antigens when the period of incorporation is prolonged. The slight difference observed by DELWICHE *et al.* seemed to these authors to be explained by

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an unequal net increase in soluble antigens and virus. We have seen that such an explanation of our results was excluded.

An interesting correlation appears possible between the hypothesis that the soluble antigens, of low molecular weight, aggregate in the cell, producing the virus particles, and the discovery that similar aggregations can be provoked *in vitro* by simple variations in salt concentration or pH of the medium[2,3,5,6]. The particles so obtained have no ribonucleic acid. They have a structure very close to that of the virus, as is shown in particular by X-ray diffraction technique[12]. They are non-infectious.

Much more striking still is a fact reported quite recently by FRAENKEL-CONRAT[13]. It has been shown previously by SCHRAMM[14,15] that a solution of his protein A, produced by degradation of the virus in alkaline solution, can be made to aggregate into particles analogous to the virus but non-infectious, simply by lowering the pH. According to FRAENKEL-CONRAT, if such an aggregation takes place in the presence of ribonucleic acid, previously isolated from the virus by a mild procedure, the particles that appear may be infectious and possess, like the normal virus, a central axis of ribonucleic acid surrounded by a sheath of protein. The reconstruction *in vitro* of a virus particle from its disintegration products may therefore be possible.

Thus our work, in providing evidence in favour of the hypothesis that soluble antigens, which are very similar to the protein A of SCHRAMM, are the direct precursors of the protein part of the virus, leads us to consider that FRAENKEL-CONRAT has effectively demonstrated *in vitro* a valid model of the final processes in the normal synthesis of the virus.

SUMMARY

Abnormal proteins which lack ribonucleic acid and are present in leaves infected by tobacco mosaic virus incorporate ^{14}C provided in the form of $^{14}CO_2$ at a much higher rate than the virus. The specific radioactivity of the virus, calculated on the basis of the hypothesis that these abnormal proteins are the immediate precursors of the protein part of the virus, is very close to the measured radioactivity. The aggregation *in vitro* of the degradation products of the virus to form infectious particles, as carried out by FRAENKEL-CONRAT, may thus be a satisfactory model of the last stage of virus synthesis *in vivo*.

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