THE ACTION OF RIBONUCLEASE ON PHAGE PROTEIN SYNTHESIS BY AN INDUCED LYSOGENIC *BACILLUS MEGATERIUM* CULTURE

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SUMMARY

Ribonuclease selectively inhibits phage protein synthesis in ultraviolet-induced lysogenic *Bacterium megaterium*.

The sooner the enzyme is added after induction, the more extensive is the inhibition. On the other hand, the number of infectious phage particles produced depends on the amount of phage protein already synthesised before RNase is added. This indicates that the phage proteins synthesized in the presence of RNase are not integrated into normal infectious particles.

Nevertheless, these proteins are assembled into phage-like particles of normal aspect containing DNA. The fact that they are not infectious seems to be related to their defective fixation onto the host bacteria.

These facts may be taken into account by a hypothesis according to which RNase modifies the structure of RNA molecules that would function as templates in phage protein synthesis.

INTRODUCTION

It is now well established that the synthesis of the protein moiety of tobacco mosaic virus is determined by the introduction into the host cell of a specific ribonucleic acid (RNA)[1-3]. The same type of phenomenon seems to occur in the case of bacteriophage, where desoxyribonucleic acid (DNA) probably initiates synthesis of the phage proteins[4]. The simplest hypothesis is that these two nucleic acids have a strictly equivalent role, that of a template on which activated amino acids line up to form the corresponding protein. Nevertheless, this simple hypothesis might not be the correct one.

Circumstantial evidence does indicate that in cellular organisms RNA molecules constitute a series of templates for protein synthesis[5,6]. On the other hand, it is certain that DNA plays a leading part in the transmission of hereditary characters and more especially in governing protein specifity. It is tempting to imagine therefore that, in these organisms, the transfer of genetic information that DNA brings to proteins passes through the intermediate of RNA. One may wonder if this might not also be the case with bacteriophage in which DNA would have a much less direct role in the synthesis of specific viral proteins than does the RNA of plant viruses[7].

This hypothesis implies that phage protein synthesis may be impaired by any

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action selectively exerted on the RNA of a phage-bacterium system during phage multiplication.

It has been shown previously that ribonuclease (RNase) inhibits synthesis of tobacco mosaic virus[8] and influenza virus[9], whose multiplication implies participation of ribonucleic acid. On the other hand, we have briefly pointed out that multiplication of a *B. megaterium* phage is also inhibited by this agent[7, 10]. This has prompted us to a more thorough investigation, the results of which are given in this paper.

The study of the action of ribonuclease on phage multiplication necessitated preliminary work on the action of this enzyme on the non infected host cell. This study has been the subject of a previous paper[11].

MATERIAL AND METHODS

The strain of *B. megaterium*, the culture techniques and the assay methods were described in a previous paper[11].

Bacteriophage production is induced by ultraviolet light[12]. At various times following induction 25 μg lysozyme (Armour) per ml of culture are added to provoke cellular lysis. The time taken for complete lysis is 2 to 5 min. Assay of infectious particles is achieved as described by LWOFF[12].

Antiserum for assay of phage-specific protein was obtained by immunizing a rabbit against bacteriophage preparations obtained by three cycles of alternate centrifugations 20 min at 10,000 rev./min and 30 min at 28,000 rev./min in the Spinco preparative rotor. The obtained antisera are treated before use by an excess of bacterial antigens in the form of non lysogenic *B. megaterium* disrupted in the Hughes press[13].

Bacterial lysates are treated with normal sera to eliminate non specific precipitates before addition of antiphage sera. Chloramphenicol was added (100 μg/ml) as an antibiotic. Immunological precipitation is allowed to proceed at 37° for an hour and at 0° overnight, after which time the precipitate is obtained by centrifugation at 10,000 rev./min for 20 min.

The precipitates are treated as indicated by KABAT[14]. DNA and total N insoluble in cold 5% trichloroacetic acid, alcohol and ether are assayed. Total insoluble N was found to be proportional to the amount of phage present in the lysates. This relation was controlled by the addition of antiphage serum to mixtures of lysates of induced lysogenic and non lysogenic *B. megaterium* in different ratios.

The same precipitation technique was used to determine the specific radioactivity of phage DNA synthesised in presence of ^{32}P-labelled phosphate. Control experiments show that the radioactivity of precipitates obtained with unlabelled phage in the presence of ^{32}P-labelled bacterial lysates is practically nil. This means that phage precipitation is very specific.

The use of this technique for the assay of phage antigen necessitated culture volume of 100 to 200 ml according to the optical density attained before lysis (0.2 to 0.4).

EXPERIMENTAL

1. *Effect of the RNase on the amount of phage protein synthesized*

a. General remarks. When lysogenic *B. megaterium* cells are induced by ultra violet light, protein and ribonucleoprotein constituents continue to be synthesized at

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a normal rate until the cells lyse and liberate phage particles. Most of these constituents belong to the host cell, and the bacteriophage accounts for only a very slight amount of the dry weight. Oxygen consumption remaining proportional to the optical density, it seems probable that these constituents are functional.

In this system, where bacterial and phage constituents are built up side by side ribonuclease can inhibit phage protein synthesis without stopping the growth of the bacterial cells. The fact that the host cells still grow normally show that RNase does not suppress essential factors of protein synthesis.

It is also easy to exclude the idea that RNase causes cell lysis which in turn would block phage synthesis. With sufficient concentrations of RNase, the normal ultraviolet-induced lysis is inhibited and growth continues slowly, no detectable amounts of phage protein appearing in the culture medium. These results were obtained with concentrations of RNase that did not lyse non-induced control cultures[11].

The technique used throughout the experiments is the following. A RNase-sensitive, lysogenic strain is lyophilized and constitutes the starting point of a series of experiments. Cultures are grown at 37° till they reach an optical density of 0.2. The culture is then induced at 30° by the smallest dose of ultraviolet light sufficient for total induction. At this moment, the culture is diluted with fresh warm medium and incubated at 30°. RNase is added at various times during the evolution of the system.

The phage proteins are assayed when their concentration has reached its maximum value. We consider that this value is obtained:

(1) in the control culture, after lysis is completed,

(2) in cultures treated with low concentrations of RNase and presenting partial lysis, when lysis reaches its maximum value,

(3) in cultures treated with high concentrations of RNase and presenting no lysis, when the increase in optical density of the culture equals that of the control culture before lysis.

b. Results obtained when RNase is added at various times following or preceding induction. A typical experiment is represented in Fig. 1. The concentration of RNase added was 100 μg/ml. Inhibition of phage protein production is total when the enzyme is added before or immediately after induction. If addition of RNase is progressively postponed, the quantities of phage formed increase to attain the normal value.

The inhibitory effect of RNase can be estimated only if we can account for the amount of phage proteins already present at the moment RNase is added[15] (Fig. 2). If we compare the amount of phage protein produced, after addition of RNase, to the amounts synthesized by the controls in the same interval, we notice (Table I) that the percent inhibition of phage protein synthesis decreases regularly with the time of addition of RNase.

The result leads to two remarks.

First, the percent inhibition decreases when RNase is added at 0, 10 or 30 min following induction, that is, during the period that precedes phage protein synthesis. During this period, RNase apparently inhibits the progressive synthesis of a necessary element for the subsequent production of the phage proteins.

In the second place, the inhibitory action of RNase, although reduced, is maintained when the enzyme is added from the 30th to the 110th minute following induction, thus during the period of accumulation of phage proteins. We may, therefore,

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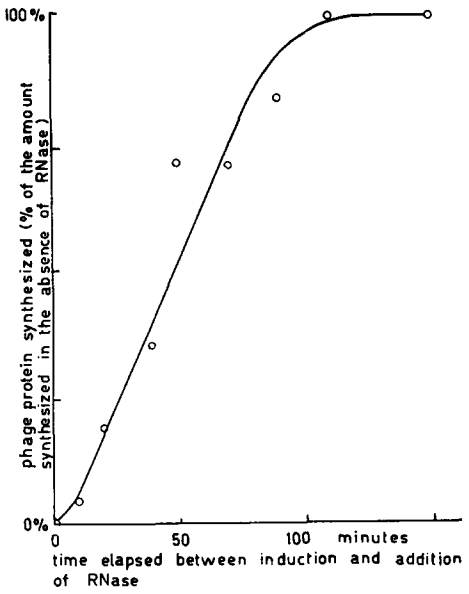

Fig. 1. Amounts of phage protein synthesized, RNase being added (100 μg/ml) at various times following induction.

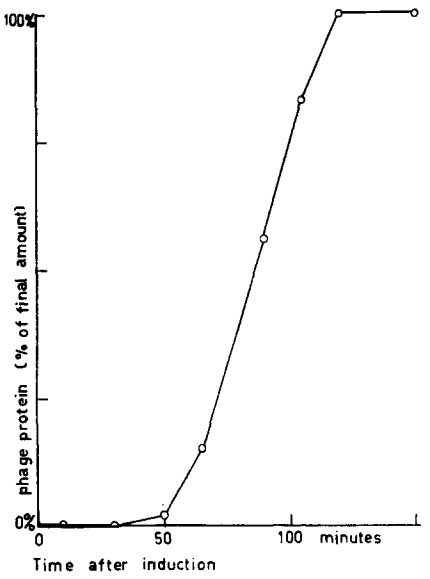

Fig. 2. Amount of phage protein found at various times following induction in lysates obtained by adding lysozyme to equal volumes of culture.

TABLE I

INHIBITION OF THE PHAGE PROTEIN SYNTHESIS AFTER ADDITION OF 100 μg/ml RNASE AT VARIOUS TIMES FOLLOWING INDUCTION

Time t (min after induction)	*Fraction of phage protein which forms after time t (as % of maximum yield in the control)*		*Inhibition of phage protein synthesis by RNase, %*
	Control (no RNase)	*RNase at time t*	
0	100	0	100
10	100	7	93
30	100	27	73
50	97.5	48	51
65	85	52.5	38
90	45	32.5	28
110	17	14	17

suppose that a ribonuclease-sensitive mechanism involved in phage protein synthesis is being built up throughout the duration of protein synthesis. If the action of ribonuclease were merely to impair the proper functioning of this mechanism, the percent inhibition would not vary with the time of addition of RNase.

c. Selective action of RNase on phage protein synthesis. The inhibitory action of RNase on bacterial proteins synthesis was studied in a previous paper[11]. Inhibition of one mechanism, common to all protein synthesis is the simplest hypothesis one can formulate concerning the action of RNase. This implies, however, that RNase must inhibit synthesis of phage and bacterial protein in the same ratio. This was never the

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case in our experiments, where the action of the enzyme on phage protein synthesis was always much more noticeable.

This selective action of RNase varies according to experimental conditions and is most apparent when the enzyme is present only during the time phage proteins are being synthesized. The results of a typical experiment, in which RNase was added at different concentrations at the beginning of phage protein synthesis (45 min after induction) are summarized in Fig. 3 and Table II. The amount of phage protein was determined as in the preceding experiments.

TABLE II

ACTION OF VARIOUS CONCENTRATIONS IN RNASE ON THE AMOUNTS OF PHAGE PROTEIN SYNTHESIZED AND ON THE NUMBER OF INFECTIOUS PARTICLES FORMED AFTER U.V. INDUCTION

Experiment as in Fig. 3.

Concentration in RNase $\mu g/ml$	*Phage protein N*	*Number of infectious particles/0.1 ml*
0	192	$1215 \cdot 10^6$
10	—	$37 \cdot 10^6$
25	54	$1.4 \cdot 10^6$
50	1.1	$0.3 \cdot 10^6$
100	1.0	$0.1 \cdot 10^6$
150	0	0

In control experiments, where protein N and optical density were simultaneously followed in the interval between addition of RNase and the beginning of lysis, it was noted that the increase in optical density constitutes a valid estimate of the amount of protein synthesized. If lysis is inhibited, the optical density measures the amount of protein N in the culture throughout the duration of the experiment.

If this is taken into account, a comparison between Fig. 3 and Table II shows that for increasing concentrations in RNase, the amount of phage synthesized is progressively reduced to nil, whilst the increase in bacterial protein, between the time of addition of RNase and lysis of the control cultures, is barely inhibited. Moreover, when lysis and phage protein production are completely inhibited by a sufficient concentraton of RNase, the amount of bacterial protein synthesized may, and often does, exceed that synthesized by the control culture. Although less striking, the selective property of RNase is apparent when it is added immediately after induction (Fig. 4 and Table III).

A more direct control of the selective action of RNase was effected under similar conditions. Total protein and phage protein was estimated at the moment phage protein synthesis starts (50 min after induction) and after it is completed (120 min after induction). In these experiments the ratio of the amount of phage protein synthesized to the total amount of protein synthesized was reduced by 75 %.

d. Action of RNase and prophage inducibility. The question arises whether the action of RNase on phage synthesis in ultraviolet-induced lysogenic *B. megaterium* might not be related to this particular type of material. The enzyme might, indeed, inhibit the transformation of the prophage into an element capable of inducing synthesis of phage constituents, without inhibiting this synthesis once it is under way.

Experiments where repeated therefore with phage-infected sensitive *B. megaterium*

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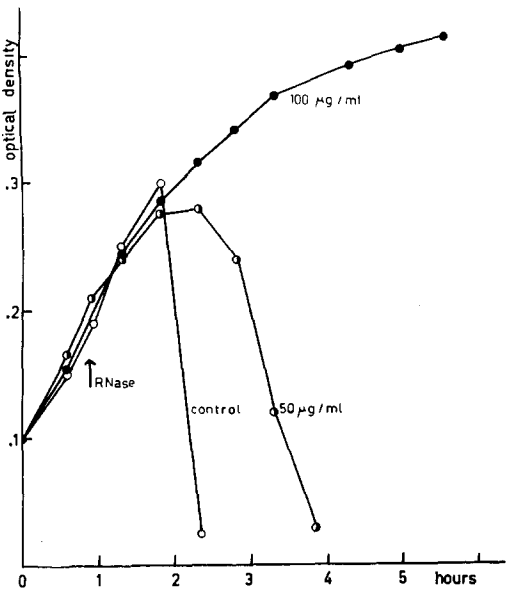

Fig. 3. Evolution of the optical density of cultures induced by ultraviolet light at the time of o hours. RNase is added at the time indicated by the arrow.

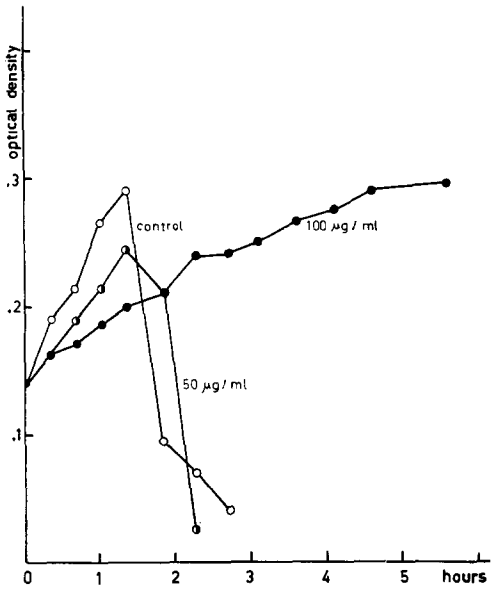

Fig. 4. Evolution of the optical density of cultures induced by ultraviolet light at the time of o hours. RNase is added immediately after induction.

TABLE III

ACTION OF VARIOUS CONCENTRATIONS OF RNASE ON THE AMOUNT OF PHAGE PROTEIN SYNTHESIZED AND ON THE NUMBER OF INFECTIOUS PARTICLES FORMED AFTER U.V. INDUCTION

Experiment as in Fig. 4.

Concentration in RNase µg/ml	*Phage protein N*	*Number of infectious particles/0.1 ml*
0	402	$283 \cdot 10^6$
50	125	$23 \cdot 10^6$
100	0	$0.5 \cdot 10^6$

to which RNase was added after infection. Infection was multiple, so as to provoke lysis of the control culture after only one multiplication cycle. The results obtained are comparable to those obtained with lysogenic bacteria. Here also lysis and phage production can be selectively inhibited; the concentrations capable of completely inhibiting lysis allow, nevertheless a cellular increase comparable to the control cultures in the same interval.

It is, thereby, apparent that the action of RNase on induced lysogenic bacteria, identical in every respect to its action on phage-sensitive infected bacteria, cannot be the result of interference with the induction process.

These experiments on phage-sensitive bacteria have allowed us to make two more observations.

First, RNase treatment has no more effect on the basophilism of infected cells than on that of non-infected cells (staining with toluidine blue[11]).

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In the second place, moderate oxidation of RNase by H_2O_2 concomitantly reduces enzymic activity (assayed *in vitro*) and inhibitory action on phage production or cell lysis. A similar parallelism between enzymic activity and inhibitory effect on growth has also been observed on uninfected cells[11].

2. *Effect of RNase treatment on the infectious character of the phage-specific material synthesised*

a. Mode of action of RNase. When the RNase is added to a culture between the moment of induction and the beginning of lysis, the number of infectious particles produced is reduced considerably more than the amount of phage-specific protein (*cf.* Table II): 25 g/ml for example, is sufficient to reduce the number of infectious particles 200-fold more than the amount of protein.

On the other hand, if the same amount of RNase is added after cell lysis and phage liberation, the number of infectious particles is not modified, even if the lysate is allowed to stand the whole night in the presence of RNase.

This excludes the possibility that RNase exerts a direct action on phage infectivity in our experimental conditions (contact between phage and RNase in solutions of high ionic concentration). The action of the enzyme on the infectivity, therefore, may be attributed only to its presence within the cells during phage synthesis and maturation.

b. Results obtained when the RNase is added at various times after induction. The infectivity of the phage material produced is largely dependent on the moment at which RNase is added.

It is striking to observe that the curve obtained from plotting the final number of infectious particles against the time at which RNase is added (Fig. 5) is identical

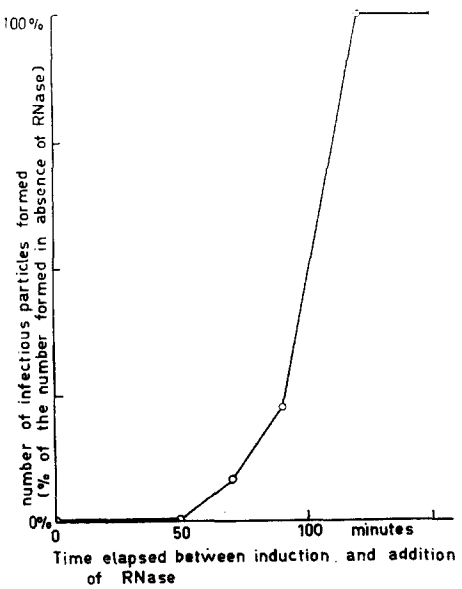

Fig. 5. Number of infectious particles produced in cultures treated with RNase at various times following induction.

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with the curve obtained by plotting phage protein synthesis as a function of time in the absence of RNase (Fig. 2).

The number of infectious particles finally produced by a RNase-treated culture appears thus to be approximately proportional to the amount of phage protein already present at the moment RNase is added.

This suggests that the phage-specific protein material, produced in the presence of the enzyme, is abnormal and unable to give rise to infectious particles; the only infectious particles are those constituted, partially at least, of material formed before addition of the enzyme.

c. Reasons for the low infectivity of phage material formed in the presence of RNase. Three different possibilities must be considered. (1) The phage proteins cannot be assembled into an infectious particle. (2) The phage particles are of an abnormal structure and incapable of injecting their DNA into the host cell. (3) The particles are either devoid of DNA or contain an abnormal type of DNA.

We have been able to gather only a small number of arguments allowing us to choose among these three possibilities. This is mainly due to difficulties experienced in obtaining abnormal material in sufficient amounts.

One particularly successful experiment was in the case of the addition of 100 μg/ml/RNase 45 min after induction. With the bacterial strain used, this concentration was low enough to permit normal increase of the culture followed by almost complete lysis. The amount of protein having phage specificity under these conditions was 36 % of that found in the control culture, whereas the number of infectious particles was only 0.94 % of that found in the control. RNase had thus decreased the infectivity per mg protein by a factor of 38.

Very small fragments of immunological phage protein precipitates of both control culture and RNase-treated culture were dried and shadowed for electron microscopy. The two precipitates appeared to be identical, being constituted of uniform particles having the size of *B. megaterium* phage heads. The phage tails were not visible, neither in the control precipitates nor in the precipitates containing the phage proteins grown in the presence of RNase. The amount of amorphous material was very slight and similar in both cases. Therefore, the reduction of the infectivity of the RNase grown material to 1/38 cannot be attributed to the fact that specific phage proteins were unable to aggregate into complex structures comparable in size to normal phage particles.

A second type of experiment was performed in which immunological precipitation was replaced by purification by several centrifugation cycles. In this experiment, the RNase-grown phage particles had an infectivity of 1/12 of that of controls obtained under the same conditions. These particles were uniform in size and appeared quite normal; the tails were apparent and of normal length. Moreover, these particles contained the normal amount of DNA.

A first attempt to find out the reason of the low infectivity of particles grown in the presence of RNase was carried out as follows:

Identical amounts of radioactive phosphate are added to two cultures, immediatly after induction. 50 min after induction, RNase is added to one of these cultures at a concentration that is low enough to permit complete lysis and abundant phage production.

Under these conditions, the specific radioactivity of the DNA phosphorus of the

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phage particles produced is equal to the specific radioactivity of the inorganic P in the culture medium[15].

This phage is resuspended after purification by centrifugation. Normal phage, and phage grown in the presence of RNase are assayed for infectious particles, protein N precipitable by antisera and DNA associated with immunological precipitates. Aliquots of these two phage solutions are added to cultures of phage sensitive *B. megaterium* of equal optical density.

After half an hour, the bacteria are spun down, washed several times, treated with trichloroacetic acid, alcohol and ether and finally dried. The DNA is isolated by the Schmidt-Thannhauser procedure, washed and hydrolysed in normal hydrochloric acid for 1 hour at 100°. Total radioactivity is determined in a solution counter tube.

Preliminary control experiments were carried out in which either ^{32}P-labelled phage plus unlabelled bacterial extracts, or unlabelled phage and ^{32}P-labelled extracts were added to phage-sensitive bacteria. These experiments showed that the radioactivity recovered in either case was derived only from the bacteriophage.

It is thus possible to determine, for each of the phage solutions, the ratio existing between the amount of phage added and the amount of phage that is irreversibly fixed on the bacteria within half an hour.

The results of a typical experiment are given below. The concentration of RNase was 15 μg/ml and did not modify either growth or lysis. The amount of phage protein was 85% of that found in the control cultures, whereas the number of infectious particles was reduced in the ratio of 193 to 33.3 for equal amounts of protein material. The amount of phage fixed on sensitive *B. megaterium*, as measured by DNA radioactivity transferred, was decreased in the ratio of 210 to 34.9 for equal amounts of phage protein added. The inhibitory effect of RNase on infectivity of phage grown in its presence is thus satisfactorily explained by the fact that a certain number of the particles are incapable of fixation onto the specific bacterial sites. This statement is based on experiments in which RNase has been used at relatively low concentrations, in order to obtain sufficient material for purification. Our conclusion is thus valid only in the case where reduction of infectivity is relatively small. It is not impossible that other lesions appear when the reduction of infectivity is considerably greater. It seems important to underline more especially that RNase, exerting its effect at very low concentration during synthesis of phage proteins, might induce a modification of their structure. This modification would convey to the protein molecules of the particle's tail, the inability to combine with the specific phage receptors of the bacterial membrane.

DISCUSSION

The inhibition by ribonuclease of bacteriophage multiplication within bacteria or protoplasts has already been reported several times[7,10,16–18].

BRENNER[19] was among the first to draw attention to the destructive action of RNase on certain bacterium–bacteriophage systems. The apparent inhibition of phage synthesis in *B. megaterium* protoplasts which he observed was solely due to a rapid and total lysis of the cells by the enzyme.

JERNE AND MAALØE[18], without observing such drastic effects, assumed, nevertheless, that in the case of *E. coli* the inhibition of phage synthesis by RNase could be the consequence of cellular disorganisation.

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It is apparent therefore that the inhibitory action of RNase on bacteriophage systems will only be significant if other biosynthetic faculties are maintained in the presence of the enzyme.

From this point of view, ultraviolet-induced lysogenic *B. megaterium* offers an advantage over the *E. coli*–T2 system, in that phage synthesis is accompanied by cellular growth until the moment the cells lyse. A control is thus supplied that biosynthetic activity continues in the host cell in presence of RNase, even though phage protein synthesis may be either inhibited or of an abnormal nature. The action of RNase, therefore, cannot be said to cause disorganisation of all the cell structures involved in protein or nucleic acid synthesis.

Valuable information concerning the possible mode of action of RNase can be gathered from our knowledge of the *E. coli* B–T_2 system. In the latter, RNA synthesis continues after phage infection, but is quantitatively very much less important than in induced *B. megaterium*[20–22]. The RNA synthesized after infection is rapidly degraded, the breakdown products being reutilized to build up phage DNA[23]. This degradation does not seem at all necessary to DNA synthesis: KIHO AND WATANABE[22] were able to suppress RNA degradation by chloramphenicol without affecting phage DNA synthesis.

Although the RNA synthesized after infection is not a compulsory precursor of phage DNA, it might, nevertheless, play an essential part in viral multiplication. HERSHEY[4] has suggested that these RNA molecules might be of a new type, necessary for the synthesis of the specific phage proteins. We have previously put forth a very similar hypothesis in which the RNA is supposed to supply the specific templates on which phage proteins are synthesized, just as for RNA in tobacco mosaic virus synthesis[7,10].

In this conception, phage DNA does not intervene directly in the determination of the phage protein specificity, but it transmits its genetic information to newly synthesized RNA. Any agent capable of selectively destroying RNA or altering its structure must also either inhibit the synthesis of phage proteins or alter their properties. This is precisely what we expected and also what we found in the case of RNase.

We have previously reported[10] that two metabolic analogues of RNA bases, 2-thiouracil and 8-azaguanine, which share the faculty of being incorporated into *B. megaterium* RNA and thereby modify its structure, exert a similar effect on page protein synthesis. Its seems most unlikely that these three agents exert the same effect by a lucky coincidence and it appears more reasonable to think that their inhibitory action is due to what they have in common, *i.e.* their influence on RNA.

It yet remains to be explained why RNase acts to a much greater extent on phage protein synthesis than on the bacteria themselves.

The phage-specific RNA (presumably newly synthesized) might be more accessible to RNase than the bacterial RNA enclosed in cellular structures pre-existing at the moment of induction. A similar situation is perhaps present in the case of tobacco mosaic virus[8] or influenza virus[9], which are sensitive to RNase only during a short period following infection.

It is also possible that RNase causes a modification in the composition of the RNA synthesised in its presence and thereby affects newly synthesized phage RNA much more than the existing bacterial RNA.

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The hypothesis in which the RNase would be active solely on those RNA molecules that are involved in the transfer of activated amino acids onto the genetic determinants (HOAGLAND) and thereby preventing protein synthesis seems of no apparent interest for this discussion. Although this hypothesis may account for an overall decrease in all protein synthesis, it is nevertheless insufficient to explain the selective action of RNase on phage protein synthesis.

A quite different explanation of the inhibitory action of RNase would be that the enzyme enhances DNase activity as it does in other cells[25]. This would imply, in our case, that RNase might indirectly exert some influence on phage DNA.

However, this hypothesis seems far from likely. First infection of a bacterium by a lytic phage also enhances DNase activity[28] without hindering phage multiplication. In the second place, it appears that phage protein synthesis can proceed either when DNA synthesis is blocked by ultraviolet light[26] or when DNA synthesis is absent as a result of a genetic modification of the prophage[27].

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