ACTION OF RIBONUCLEASE ON NEOPLASTIC GROWTH

II. ACTION ON LANDSCHÜTZ ASCITES CELLS *IN VITRO*

by

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In a previous communication[1], the *in vitro* action of ribonuclease (RNase) on ascites cells, has briefly been described. As it is known, the ascites tumour cells are suspended in a nutritive fluid with which they exchange readily metabolic intermediates. The study of ribonuclease action on such a system thus requires parallel observation of the cells and of the fluid itself. Experiments with normal ascites tumours[2] reveal that aging of the tumour has an effect on both the cells and the external fluid (ascitic plasma); the intracellular RNA content decreases by 50% during the growth while no appreciable change occurs in the DNA, protein or free nucleotide content; in the ascitic plasma, the contents in RNA, protein and free nucleotides increase during this period.

In studying the action of RNase, we therefore have to take into account the actual physiological age of the suspensions used and their chemical constituents. Another factor which must be carefully considered is the following: in both intercellular and extracellular fluids, there exist equilibria, e.g. between RNA and nucleotides, and these might be influenced by the action of the added enzyme. The dynamic equilibria which are set up between cells and the ascitic plasma also will be disturbed if the enzyme acts on the external constituents. On the other hand, it is probable that the penetration of protein into the cells depends on "permeability" mechanisms which could vary qualitatively or quantitatively during the aging of the tumour. In consequence, the quantity of enzyme penetrating the cells, per unit time, could vary, and obviously would then influence variably the observed phenomena.

In this connection one has to remember the recent observations of HEPPEL *et al.*[3,4] who have shown that RNase, being partly a phosphotransferase, is also capable of anabolic activities at low concentrations. Thus, the presence in the cell of different amounts of enzyme could induce anabolic or catabolic processes.

The present paper, which deals with the *in vitro* action of RNase on ascites tumour cells at different moments of their growth, describes results obtained by chemical methods only. Results obtained by other methods will be described later.

MATERIALS

The tumour

During this study, Landschütz ascites tumours of different rates of growth have been used. One type of cells, originating from KLEIN's laboratory, gave with the C^+ mice used, medium survival

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times (MST) of about 7 days (when implanting 10^7 cells). It will be referred to in this paper as the "quick growing" type.

Another type of cells was produced by repeated transfers of old ascites suspensions (12–13 days) taken from mice treated with the "quick-growing" type of cells, but having survived longest. After several transfers, a type of cell was obtained which produced the death of the animals later on: MST = 14 days for implantation of 10^7 cells. This type of cell will be called here "slow-growing" type. Fig. 1 shows typical mortality curves of these tumours.

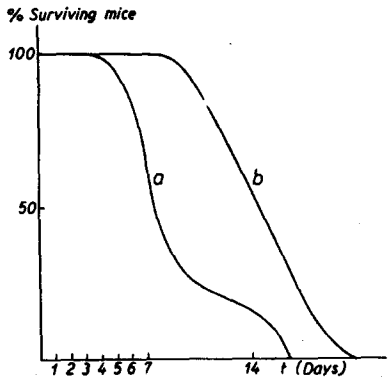

Fig. 1. Mortality curves for Landschütz ascites tumours. Curve a, quick growing type; Curve b, slow growing type.

The reasons for this apparent selection could lie in the fact, as suggested by KLEIN[5], that this tumour is still not entirely non-specific from the immunogenetic point of view and that there could be some incompatibility between the tumour and the C^+ strains used here.

Products

Ribonuclease: 1. Armour, GBI, Worthington (pancreatic ribonuclease); 2. freshly purified RNase prepared in the Institute from beef pancreas (by Mr. P. G. AVIS, to whom the author's thanks are due), and chromatogrammed[6]. The oxidised RNase was obtained by oxidising the product with catalysed hydrogen peroxide, evaporating the solutions then *in vacuo*, and destroying the hydrogen peroxide left, with small amounts of catalase.

Ribonucleic acid (RNA): Hopkins and Williams preparation purified by reprecipitation and dialysis.

Deoxyribonucleic acid (DNA): Thymus DNA obtained from calf thymus and purified.

Protein: Ribonuclease purified by chromatography[6] was also used as standard protein.

METHODS

The ascitic fluid was withdrawn with a hypodermic syringe, fitted with a No. 12 needle. The sample was transferred as quickly as possible into incubation tubes containing a determined amount of RNase which was, in most of the cases, dissolved in the smallest possible amount of saline or of ascitic plasma. The contents of the tubes were then mixed; in most cases, the tubes were gently shaken throughout the incubation.

At given times, a sample was withdrawn from the tube and centrifuged at once at 3.500 r.p.m. from 3 min. The supernatant was separated and stored.

Both cells and supernatant were then treated according to SCHNEIDER[7] and different fractions were obtained; acid-soluble ("free-nucleotides"), TCA extract (DNA and RNA) and proteins. The acid-soluble was analysed by the U.V. spectrophotometric method, using the difference $E_{260}-E_{300}$ as a measure of the free nucleotide content. Numerous tests have proved that the results so obtained were identical with those obtained by the orcinol colorimetric method[7].

The TCA extract was analysed according to SCHNEIDER: for RNA by using the modification of LUSENA[8], for DNA by using the usual technique[7].

The proteins were determined by the application of MEHL's method[9] after digestion of the TCA residue by *N* KOH.

RESULTS

Results obtained with different brands of RNase

When using, in parallel experiments, different preparations of RNase, with samples of ascitic fluid withdrawn from the same animals, very different results could be obtained. These facts have already been indicated previously[10].

It should be emphasised that the GBI, Worthington, and freshly prepared RNase had very similar effects on ascites cells; they were capable, in certain circumstances, of producing net increase of intracellular RNA. This never occurred with the Armour preparations tried so far. Even preparations purified by chromatography showed similar differences. Fig. 2 illustrates effects obtained in a typical experiment.

The reasons for these differences could be due to differences in molecular structure

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or amino acid composition. It is now proved[11,12,13] that the same enzymic activity can be obtained from variously degraded ribonucleases.

Whatever the real reason, these facts prompted us to use a well defined preparation. We, therefore, chose (and used in the experiments described below) the chromatogrammed fraction having the highest specific activity[6] isolated from a freshly prepared pancreatic RNase (peak A)[6].

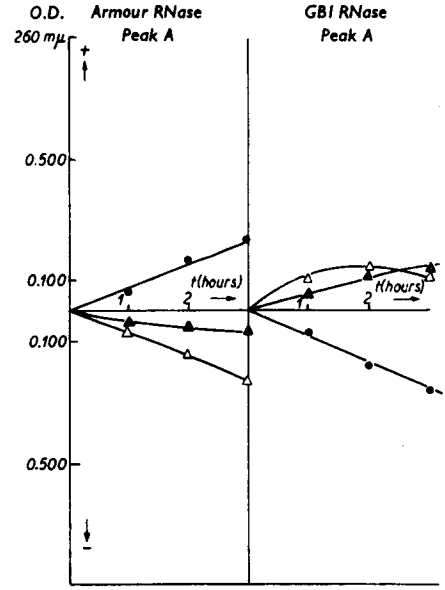

Fig. 2. Action of different RNase on same tumour cells (1 mg/ml). △ RNA intracellular; ▲ Free nucleotides intracellular; ● Free nucleotides extra cellular.

Results obtained with the quick-growing Landschütz tumours

1. *Young tumours* (physiological age: 1–4 days).

RNase produces in most cases (4 out of 5 experiments) a significant decrease of the RNA/DNA ratio accompanied by a slight decrease in the protein content. Fig. 3 shows typical results for three selected temperatures of incubation: 17°, 27° and 37°. Samples of the same ascites cells suspension were used in each case.

In the controls, made up with an equivalent concentration of oxidised RNase, no change occurred under these conditions, up to 8 hours. After this time, the intracellular concentrations began to decrease slowly.

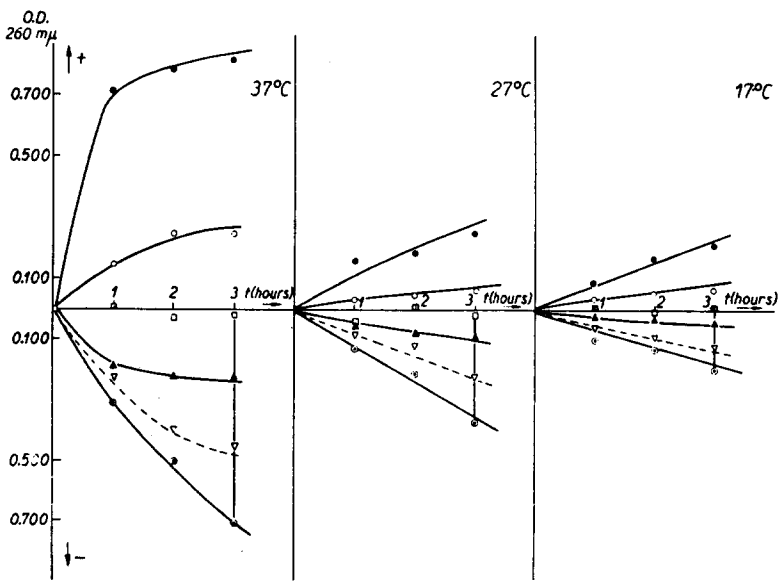

Fig. 3. Action of RNase on Landschütz ascites cells *in vitro* (quick growing type–young tumours). ◉ RNA intracellular; ▲ Free nucleotides intracellular; □ DNA intracellular; ▽ Protein intracellular; ● Free nucleotides extracellular; ○ RNA extracellular; RNase = 2 mg/ml fluid.

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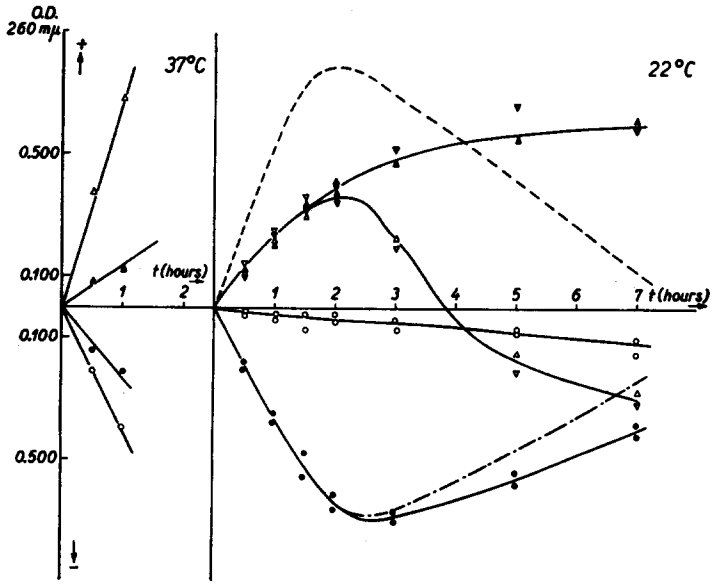

Fig. 4. Action of RNase on Landschütz ascites cells *in vitro* (quick growing type–old tumours). △ RNA intracellular; ▲ Free nucleotides intracellular; ○ RNA extracellular; ● Free nucleotides extracellular; ----- Sum of constituents appearing in cell; -·-·- Sum of constituents disappearing in the external fluid. RNase = 2 mg/ml fluid.

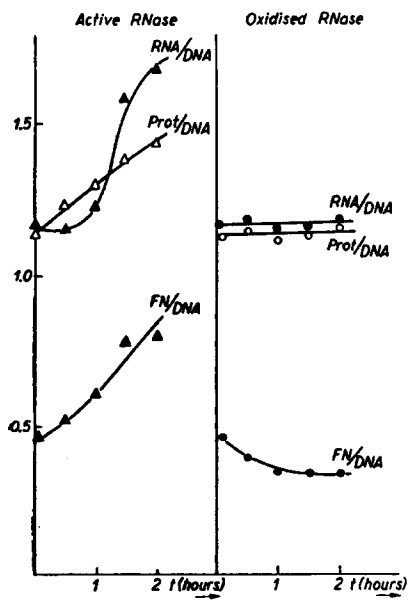

Fig. 5. Action of RNase on Landschütz ascites cells *in vitro* (quick growing type – old tumour). RNase = 5 mg/ml fluid. Intracellular constituents.

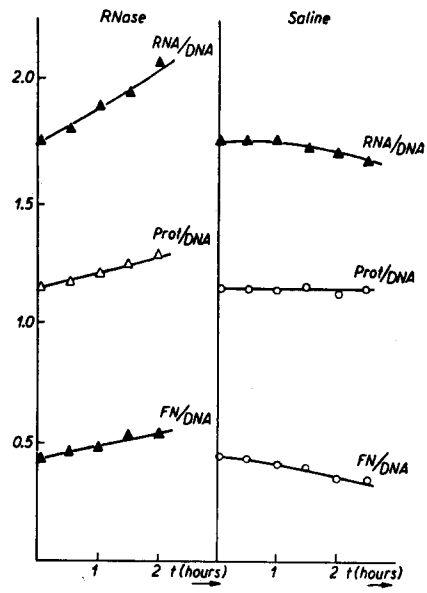

Fig. 6. Action of RNase on Landschütz ascites cells *in vitro* (slow growing type – young tumours). RNase = 5 mg/ml fluid. Intracellular constituents.

2. *Old tumours* (physiological: 5–16).

RNase produced in most cases (4 out of 5 experiments) an increase of the RNA/DNA ration in one h. This increase was accompanied by similar increase of the protein content. Figs. 4–5 show typical results.

It has been emphasised that the increase of internal RNA concentration is always accompanied by a significant increase of the free nucleotide content of the cells.

In the controls, made up with oxidised RNase, no change occurred: in controls made with saline alone, there occurred a slight decrease of the cellular content. From Fig. 3, one can see that the different constituents are actively exchanged between the cell and the surroundings.

Results obtained with the slow growing Landschütz tumour

1. *Young tumour* (physiological age: 1–4 days).

RNase produced in 4 experiments out of 5, significant increase of RNA/DNA ratio in one h. Fig. 6 shows typical results.

Here too, the protein and RNA content seemed to vary in the same way.

2. *Old tumours* (physiological age: 5–18 days).

RNase produced in 4 experiments out of 8, an increase of RNA/DNA ratio in two h. In the 4 other cases, no changes at all occurred during this period. In the controls (oxidised RNase or saline), the intracellular content remained unchanged or decreased somewhat. The results obtained here were qualitatively similar to those of the preceding section (C1).

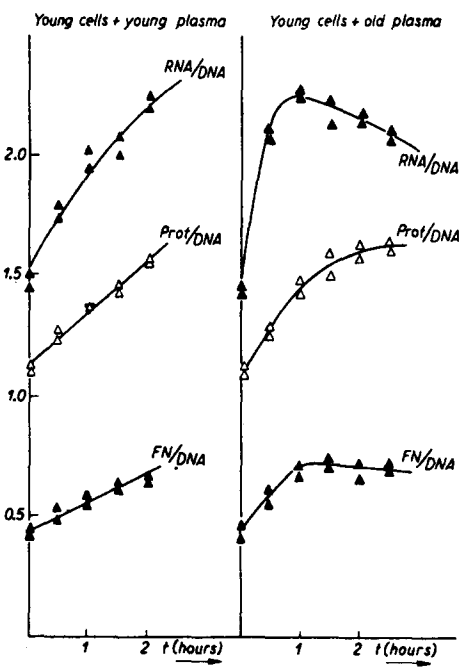

Fig. 7. Action of RNase on Landschütz ascites cells *in vitro* (slow growing type – young cells + young plasma, and young cells + old plasma). RNase = 5 mg/ml fluid. Intracellular constituents.

3. *Young tumour cell + external fluids of various age*

To test the relative importance of the age of cells and fluid, we chose active young cells (withdrawn from a slow growing tumour 3 days old) centrifuged them at low speed and placed them either in their own plasma, or in a fluid taken from a centrifugated suspension of ascites tumour, 12 days old.

The two suspensions were then gently mixed and the same amount of RNase was added to each of them (5 mg/ml fluid). Fig. 7 shows the results obtained.

It appears from these data, that the age of the plasma was important for the rate of increase of RNA/DNA or Prot/DNA ratios. Presumably, the old plasma was enriched[2] in constituents necessary for the RNase action.

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4. *Old tumour cells (13 days) + external fluids of various ages*

Ascites cells 13 days old were placed in plasma taken from a centrifuged suspension of young cells (implant 4 days old). As comparison we used the 13 days old cells in their own fluid. Fig. 8 indicates the results obtained.

Here again, the presence of old fluid increased the rate of the RNA/DNA changes.

When compared with the previous results (C3), the extent of the modifications appeared to be much smaller. However, the fluids used in both these experiments were taken from the same animals at one day intervals.

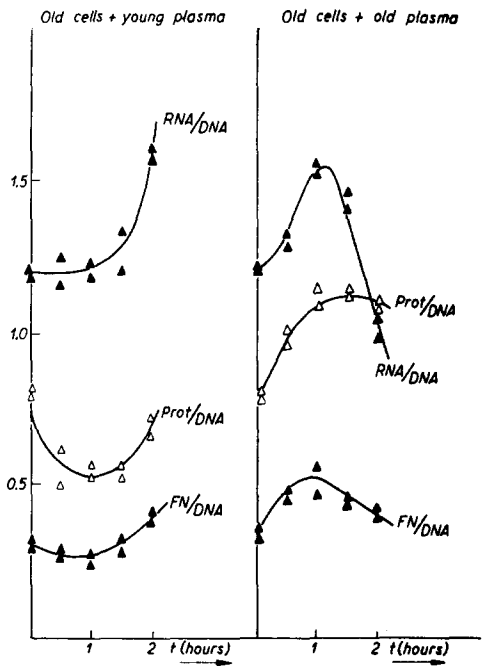

Fig. 8. Action of RNase on Landschütz ascites cells *in vitro* (slow growing type – old cells + young plasma, and old cells + old plasma). RNase = 5 mg/ml fluid. Intracellular constituents.

DISCUSSION

Growth rate of the tumour

The rate of growth of the tumours *in vivo* has a direct influence on the action of the RNase *in vitro*: indeed, it is possible to observe either an increase or a decrease of the intracellular content according to the physiological state of the suspensions.

Generally speaking, the modification of the ratio RNA/DNA observed in one or the other case are associated with similar variations of the ratios free nucleotide/DNA and Protein/DNA.

In none of the cases, similar variations have been observed in absence of enzymically active RNase.

When the tumour used belonged to the "quick growing" type, it was possible to obtain, during the first part of the RNase action, rapid increase of the ratio RNA/DNA when the physiological age of the suspension used was high*.

With young and very active suspensions, one generally obtained a rapid decrease of the RNA/DNA ratio, frequently associated with a disruption of the cells (during the last part of the action of RNase). When the tumour used belonged to the "slow growing" type, it was possible to obtain a significant increase of different cellular constituents (*per unit DNA*) throughout the tumour growth, but the results were less reproducible with old suspensions than with young ones. They give the impression that, in these cases, the enzyme did not always penetrate into the cells.

Penetration of RNase into living cells

Microscopical observations[24] indicate that the cells ability to form pseudopods can be related to the effectiveness of the ribonuclease action: in almost all cases

* In these suspensions, the actuai rate of growth[22], the rate of glycolysis[23], the pseudopoidal movements[24] and the RNA content[2] were low.

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where no significant effect was observed, the cells seemed mechanically inert[24]. This suggests that the penetration of the enzyme could be mainly due, here, to a pinocytosis mechanism. It has already been shown that the ribonuclease can penetrate the cells without impairing its vital activities; in the case of onion root tips, KAUFMANN AND DAS showed that there are mitotic abnormalities after treatment of the intact roots with solutions of this enzyme[14] and BRACHET proved that these modifications are accompanied by various changes in cellular content[15]. He also showed that ribonuclease penetrates the amoebae[16] while JEENER observed a similar effect on tobacco leaves[17]. On the other hand, we previously described some effects of the penetration of ribonuclease into intact amphibian eggs[18, 19]. By using labelled RNase, we then observed that the active and inactive forms of this enzyme penetrated the cells at the same rate. The penetration process therefore seems not to be due to an enzymic action on the membrane[20]. This was confirmed, in the case of root tips, by the findings of KAUFMANN AND DAS, that the rate of movement of ribonuclease across cellular membranes have an optimum pH quite different from that of its enzymic action[21].

Interaction cell-ascitic plasma

The parallel study of the changes in intra- and extra cellular contents revealed that these two media were rapidly and continuously exchanging and that the actual content of the extracellular fluid directed, in some way, the action of the enzyme.

In fact, the increase of the intracellular RNA content was accompanied by a depletion of the acid soluble from the extracellular fluid. Seemingly, in most cases, the RNA appearing in the cells was built, *in situ*, from precursors in the external fluid.

The observed accumulation of RNA inside the cell was not, generally, accompanied by an equivalent disappearance of the external RNA. In the presence of the RNase there occurred a synthesis of RNA. Because this synthesis was only observed in tumour cells of relatively low mechanical activity, it is not unlikely that this synthesis only occurred at low concentrations of the enzyme. At higher concentrations, the catabolic action seemed to predominate*. This fact might be related to the observations of HEPPEI *et al.*[3, 4] on the anabolic *in vitro* action, of RNase and also to the results of HOKIN AND HOKIN on the activating action of RNase on RNA metabolism[25].

The important role played by ascitic plasma was also demonstrated by the fact that plasmas from old tumours added to suspended cells exposed to RNase accelerated the effects of the enzyme on the cellular constituents. It appears therefore that during the aging of the tumours, an enrichment of the plasma in precursors of RNA[2] takes place. Such experiments with cells of various physiological ages, associated with plasmas of different ages, suggest moreover that the *occurrence* of increase of cellular content (per unit DNA) depends on the physiological state of the cells, while the *rate* of this increase mainly depends on the extracellular fluid. This is also in agreement with the observation made on tumour cells of the "quick growing" type**.

* It must be emphasised that all the present determinations were balance measurements of the equilibrium: RNA $\rightleftharpoons$ precursors (or products).

** It is known[22] that the growth rate continuously decreases during the development of the tumour.

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Use of DNA as base line reference

RNase often produces disruption or clotting of the tumour cells, so that it is generally impossible to express the results per unit volume of suspension. This makes the use of reference substance necessary. We have chosen DNA because this constituent seems *a priori* to be very constant; on the other hand, RNase is known to have an effect on the synthesis of proteins[26-27], so preventing the use of protein content as reference.

However, it remains to be established beyond doubt that some of the observed changes in different ratios are not partially due to variations of DNA content.

This is now being studied by other methods, but so far it appears reasonable to adopt the present procedure.

Action of RNase on the synthesis of protein

The increase of the ratio Protein/DNA, generally observed in presence of the RNase during the first part of its action on the cells does not necessarily represent a real protein synthesis. Our data are still insufficient to distinguish between a synthesis and an intake of protein due to modifications of cell permeability.

However, the increase in RNA content seems to be correlated with a parallel increase of protein concentration; this is in fair agreement with the hypothesis of BRACHET[28] and CASPERSSON[29].

Concerning the nature of the RNA synthesised in these *in vitro* experiments with RNase, we have recently obtained indications of differences between this RNA and that normally present in the cells*. This needs further investigation.

In any case, the presence of RNase in suspensions of ascites cells causes, *in vitro*, an important modification of cellular metabolism. This is probably responsible for the anti-tumour effects of this enzyme observed *in vitro* with ascites[30] and solid tumours[31].

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SUMMARY

The study of the *in vitro* action of ribonuclease, on a series of suspensions of ascites cells in different physiological conditions, shows that the activity of the cellular membrane and the constitution of the ascitic plasma directs the observed phenomena.

An important initial increase of cellular components (synthesis of RNA, increase of proteins and of free nucleotides) is frequently observed and the *occurrence* of this phase depends on the membrane activity (pseudopoidal movements) of the cells.

The *rate* and the *importance* of these changes depend on the constitution of the extracellular fluid.

This modification of the cellular metabolism is probably responsible for the anti-growth effect of the ribonucléase.

* *Note added in proof*: Evidence has been obtained (by using labeled compounds) that the process of incorporation of purine and pyrimidine precursors is differently affected by RNase. These results and others, to be published, suggest that synthesized RNA is enriched in pyrimidines and that the turnover of these substances is greatly increased.

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