CCA-514

577.15:547.963.32
Original Scientific Paper

The Possible Roles of Known Enzymes in the Breakdown of RNA in X-Irradiated *Escherichia coli*

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Received April 12, 1968

Breakdown of ^{14}C-adenine-labelled RNA of X-irradiated *Escherichia coli* proceeds at the same rate in the presence and in the absence of chloramphenicol, thus indicating that pre-existing enzymes are responsible for the process. Though considerable amounts of RNA are broken down upon the incubation of bacteria in some salt solutions, the breakdown is enhanced by irradiation only when the incubation medium contains both K^+ and phosphate. Nucleoside diphosphates were found among the breakdown products of ^{14}C-adenine and ^{14}-uracil-labelled RNA, together with larger quantites of nucleoside monophosphates and some triphosphates. The radiation-induced breakdown of RNA in a strain lacking ribonuclease I, *E. coli* MRE 600, proceeds at the same rate as in *E. coli* B. On the basis of these and some earlier findings it is proposed that ribonuclease I does not take part in degradation, while both ribonuclease II and polynucleotide phosphorylase are responsible for the process.

To a number of treatments causing the breakdown of bacterial RNA we have recently added the exposure to ionizing irradiation[1,2]. The breakdown starts some 30 min. after irradiation and its final degree is dose-dependent[1]. It is characterized by a high rate as compared to the rates of degradation during nitrogen[3], phosphate[4], or magnesium deficiency. It is possible to control the rate of breakdown by changing the simple physical parameter, *i. e.* the dose of irradiation[2]. The degradation is accompanied by concomitant net synthesis of RNA in the bacterial culture and affects ribosomal RNA, leading to the formation of high-molecular-weight intermediates. The main breakdown products of adenine-labelled RNA formed during the incubation in mineral medium were identified as AMP* and GMP (ref. 2). These data lead us to the conclusion[2] that ribonuclease I (EC 2.7.7.16) probably did not take part in the formation of final breakdown products, though its role in the initial stages of degradation was not excluded. The possible roles of other two degradative enzymes, ribonuclease II (EC 3.1.4.1) and polynucleotide phosphorylase (EC 2.7.7.8), remained unknown. The availability of a strain of *E. coli* with no ribonuclease I activity[6], as well as our finding that the breakdown could be modified by various post-irradiation treatments, facilitated our investigation of the roles which might be played by the known enzymes of *E. coli* in the

* The following abbreviations are used: RNA, ribonucleic acid; DNA, deoxyribonucleic acid; AMP, GMP, UMP, and CMP, adenosine-, guanosine-, uridine- and cytidine-5′-monophosphates; ADP, GDP, UDP and CDP, corresponding nucleoside diphosphates; ATP, GTP, UTP and CTP, corresponding nucleoside triphosphates.

breakdown of RNA after irradiation. The results described in the present paper show that ribonuclease I is not involved in the process, while both ribonuclease II and polynucleotide phosphorylase are likely to be responsible for degradation.

MATERIALS AND METHODS

Bacteria and Growth Conditions

E. coli B was used in majority of the experiments. *E. coli* MRE 600, used in some experiments, was kindly supplied by Dr. H. E. Wade, Microbiological Research Establishment, Porton, nr. Salisbury, Wilts., England. This strain is reported to display negligible ribonuclease I activity[6,7]. We compared ribonuclease I activity of the two strains by using assay A of Gesteland[7], and found in *E. coli* MRE 600 less than 0.1% of the activity present in *E. coli* B. Both bacterial strains were grown in the mineral medium described earlier[2]; the generation time was about 40 min. for the strain B and about 50 min. for MRE 600.

Analysis of Breakdown Products

Radioactive breakdown products of ^{14}C-adenine and ^{14}C-uracil-labelled RNA were first fractionated on a column of Sephadex G-25 as described previously[2]. Appropriate fractions were then pooled and analysed by paper chromatography. In addition to the solvent systems used earlier[2] the following systems were employed: solvent 1, ethanol — 1 *M* ammonium acetate, *p*H 5 (75 : 30, v/v)[8]; solvent 2, isobutyric acid — 1 *M* NH_4OH (100 : 60, v/v)[9]; solvent 3, water adjusted to *p*H 10 with NH_4OH (ref. 10); solvent 4, 70 ml. *tert*-butanol, 13.2 ml. constant boiling HCl, water to 100 ml.[11]; solvent 5, isopropanol — conc. HCl—water (170 : 41 : 39, v/v)[12]. Solvents 1 and 2 were used for the separation of phosphorylated derivatives of nucleosides, and the other solvents for the separation of bases.

Other Methods and Materials

All other procedures, such as X-irradiation, sampling, determination of radioactivity of bacterial RNA, and counting of the strips of paper chromatograms were described previously[2].

^{14}C-adenine (specfic activity, 53.6 mCi/mmole) and ^{14}-C-uracil (6.5 mCi/mmole) were products of Schwarz BioResearch, Inc., Orangeburg, N.Y.

RESULTS

Breakdown in the Presence of Chloramphenicol

In all our experiments, independently of the dose of irradiation, the breakdown of RNA started after a lag of about 30 min.[1,2]. Obviously, during this period a number of processes responsible either for triggering or catalizing the breakdown could take place. To see whether the synthesis of a new protein is needed during this lag, we investigated the kinetics of breakdown in the presence of chloramphenicol. The results of such an experiment (Fig. 1) show that the inhibition of protein synthesis during post-irradiation incubation does not influence the rate of breakdown; it only extends the 30 min-lag period in non-treated irradiated culture to about 60 min. in the irradiated culture treated with chloramphenicol. This result, and the fact that RNA is also broken down when bacteria are incubated in some salt solutions (see below) show that pre-existing nucleases are responsible for degradation. At the moment, the elongation of the lag period by chloramphenicol remains unexplained.

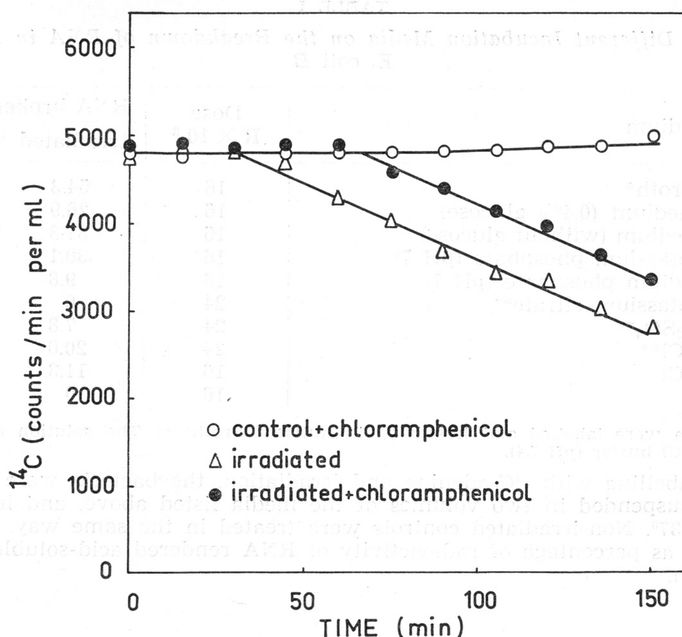

Fig. 1. Breakdown of RNA of *E. coli* B incubated in the presence of chloramphenicol after the irradiation. ^{14}C-Adenine was added to an exponentially growing culture of *E. Coli* B in mineral medium to a final concentration of 2×10^{-7} *M*. After 40 min. the bacteria were centrifuged, washed and re-suspended in mineral medium containing non-radioactive adenine. After 5 min. of incubation at 37°, one part of the bacterial culture was irradiated with 24000 R. Both parts were then diluted with equal volumes of the fresh medium; to the control culture and to the part of the irradiated culture chloramphenicol was added to a final concentration of 10 μg/ml. The cultures were then incubated at 37°. 1 ml. samples were taken for the determination of radioactivity in RNA fraction.

Influence of Different Incubation Media on the Breakdown of RNA

It has already been reported that the degradation of RNA in X-irradiated *E. coli* takes place when the bacteria are incubated in nutrient broth or in a mineral medium containing glucose[2]. To learn more about the requirements for the breakdown we performed a number of experiments in which irradiated bacteria were incubated in different non-nutritive media, *e. g.* in some salt solutions. The results, listed in Table I, show again a marked breakdown of RNA in two nutrient media (cf. ref. 2). In these media there is no breakdown in non-irradiated controls. Omission of glucose from the mineral nutrient medium leads to some degradation in the control bacteria; in irradiated culture the degradation is markedly enhanced. The same enhancement is observed when irradiated bacteria are incubated in 0.066 *M* potassium phosphate buffer (*p*H 7). On the contrary, in the solutions of some other potassium salts, as well as in sodium phosphate and chloride, the breakdown is not enhanced by irradiation, though it is quite pronounced in some of the non-irradiated controls. It seems, therefore, that the simultaneous presence of both K^+ and phosphate is indispensable for the radiation-induced breakdown of RNA in *E. coli.*

To ascertain that the need for phosphate just described is not a fortuitous consequence of the salt concentration worked with, we examined the breakdown of RNA at various concentrations of potassium phosphate and KCl. The

TABLE I

Influence of Different Incubation Media on the Breakdown of RNA in X-Irradiated E. coli *B*

Medium	Dose R × 10^{-3}	RNA broken down (%)	
		Irradiated	Control
Nutrient broth*	16	54.3	0
Mineral medium (0.4% glucose)	16	26.9	0
Mineral medium (without glucose)	16	31.3	6.5
0.066 *M* potassium phosphate (*p*H 7)	16	30.1	9.1
0.066 *M* sodium phosphate (*p*H 7)	16	9.8	11.2
0.066 *M* potassium citrate**	24	0	0
0.066 *M* K_2SO_4**	24	7.8	6.6
0.066 *M* KCl**	24	20.0	20.0
0.15 *M* NaCl	16	11.3	10.1
H_2O	16	0	0

* Bacteria were labelled and irradiated in nutrient broth; ** The solution contained also 0.005 *M* Tris-HCl buffer (*p*H 7.4).

After labelling with ^{14}C-adenine and irradiation, the bacteria were centrifuged, washed, re-suspended in two volumes of the media listed above, and incubated for 150 min. at 37°. Non-irradiated controls were treated in the same way. Degradation is expressed as percentage of radioactivity of RNA rendered acid-soluble at the end of incubation.

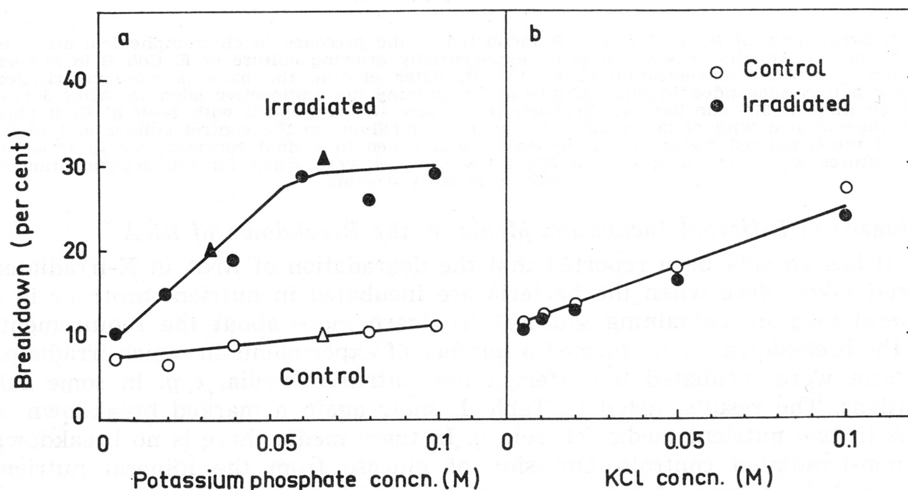

Fig. 2. Breakdown of RNA at various concentrations of (*a*) potassium phosphate buffer (*p*H 7) and (*b*) KCl. A culture of *E. coli* B growing exponentially in mineral medium was labelled with ^{14}C-adenine (2 × 10^{-7} *M*); 40 min. later one half was irradiated with 24000 R. 10 ml. samples were then centrifuged, the bacteria were washed with 0.005 *M* Tris-HCl (*p*H 7.4), and re-suspended in 20 ml. of (*a*) potassium phosphate (*p*H 7) and (*b*) KCl of various concentrations. The solutions of KCl contained also 0.005 *M* Tris-HCl buffer (*p*H 7.4). After 150 min of incubation at 37°, 1 ml. samples were taken from all cultures for determination of radioactivity in RNA fraction. The two symbols in Fig. 2a (triangles and circles) indicate the values obtained in two independent experiments.

breakdown was dependent on the concentrations of both salts (Fig. 2); its enhancement by irradiation was observed at all examined concentrations of potassium phosphate and at none of the examined concentrations of KCl.

By additional experiments it was shown that the kinetics of radiation-induced breakdown in potassium phosphate and in our mineral medium without glucose (containing also potassium phosphate) displayed a 30 min. lag, similar to that in complete nutrient media (cf. Fig. 1). The breakdown of RNA in KCl as well as in other salt colutions listed in Table I proceeded at a linear rate, which was equal for both irradiated and control culture, and without any lag.

The requirement for K^+ was further examined by incubating irradiated bacteria in 0.066 *M* phosphate buffers containing different molar ratios of K^+ and Na^+; not only was K^+ required for radiation-induced breakdown, but Na^+ exerted an inhibitory effect (Fig. 3).

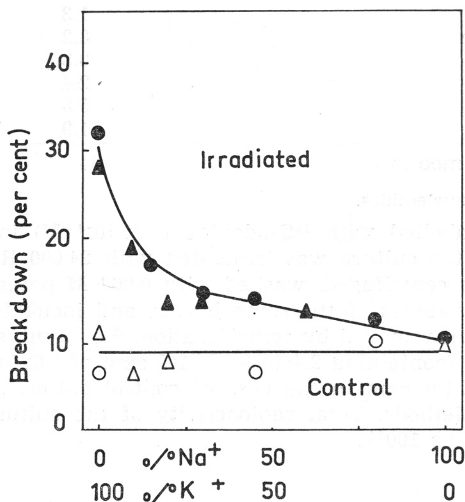

Fig. 3. Breakdown of RNA in phosphate buffers containing various molar ratios of K^+ and Na^+. To an exponentially growing culture of *E. coli* B in mineral medium ^{14}C-adenine was added to a final concentration of 4×10^{-7} *M;* 40 min. later one half on the bacterial culture was irradiated with 20000 R. 10 ml. aliquots were then centrifuged and re-suspended in 20 ml. of 0.066 *M* phosphate buffer (*p*H 7) containing various molar ratios of K^+ and Na^+. All cultures were then incubated for 150 min. at 37°; 0.5 ml. samples were taken for determination of radioactivity in RNA fraction. The two symbols (triangles and circles) indicate the values obtained in two independent experiments.

Analysis of Final Breakdown Products

As shown previously AMP and GMP are the main breakdown products of ^{14}C-adenine-labelled RNA, if the bacteria are incubated after the irradiation in mineral medium containing glucose[2]. It was of interest now to confirm that the pathway of breakdown of RNA in bacteria incubated in potassium phosphate was similar to that in the complete medium. For this reason we performed a chromatographic analysis of the breakdown products of ^{14}C-adenine-labelled RNA after incubating irradiated bacteria in 0.066 *M* potassium phosphate. In addition to AMP and GMP, the corresponding diphosphates and triphosphates, and some free purine bases were also found (Table II). It is possible that free bases were also formed in the complete medium, but were not detected because of their re-incorporation into bacterial nucleic acids.

Another search for nucleoside diphosphates in the complete medium was undertaken using ^{14}C-uracil to label pyrimidine nucleoside derivatives. As

TABLE II

Breakdown Products of ^{14}C-Adenine-Labelled Nucleic Acids After the Incubation of X-Irradiated E. coli *B in Phosphate Buffer*

Product	Radiactivity of the medium (% of total)	
	Irradiated	Control
ATP	0.9	0
ADP	1.8	0
AMP	10.9	0.5
Adenine	0	0.8
Inosine	1.3	0.7
Hypoxanthine	8.2	2.9
GDP	0.8	0
GMP	2.4	0
Guanine	5.8	3.2
Unidentified*	1.9	0.9
Total in the medium	34	9

* Presumably oligonucleotides.

E. coli B was labelled with ^{14}C-adenine (5×10^{-6} *M*) in mineral medium for 40 min. One half of the culture was irradiated with 24 000 R in the same medium. Both parts were then centrifuged, washed with 0.066 *M* potasium phosphate (*p*H 7), resuspended in two volumes of the same buffer, and incubated for 150 min. at 37°. The bacteria were then removed by centrifugation, 6 or 9 ml. samples of the medium concentrated to 1 ml. (containing 2.4 to 3.6×10^5 cpm of ^{14}C in the case of irradiated culture, and 6 to 9×10^4 cpm in the case of control culture), and further analysed as described under Methods. Total radioactivity of the culture at the beginning of incubation was taken as 100%.

TABLE III

Breakdown Products of ^{14}-C-Uracil-Labelled Nucleic Acids After the Incubation of X-Irradiated E. coli *B in Mineral Medium*

Product	Radioactivity in the medium (% of total)
UTP	2.0
UDP	2.7
UMP	10.7
CTP	2.3
CDP	2.8
CMP	9.5
Uracil	0.9
Cytosine	0.7
Thymine	3.8
Unidentified	0.6
Total in the medium	36.0

E. coli B was labelled with ^{14}-C-uracil (10^{-5} *M*) in mineral medium for 40 min. The culture was then irradiated with 24 000 R, centrifuged, washed, re-suspended in two volumes of complete mineral medium, and incubated for 150 min. at 37°. The bacteria were removed by centrifugation, and the medium analysed as described under Methods. Total radioactivity of the culture at the beginning of incubation (2.2×10^5 cpm) was taken as 100%.

expected, significant amounts of CDP and UDP were found in the medium (Table III), together with some CTP and UTP. Corresponding monophosphates were again the main products of degradation. In addition, an appreciable amount of thymine was detected, obviously originating from DNA, which is also broken down in X-irradiated *E. coli*[13,14]. However, the occurrence of breakdown products of DNA in the medium does not interfere with the conclusion that ribonucleoside diphosphates and triphosphates are formed by the breakdown of RNA. The triphosphates are probably formed (at least during the incubation without energy source, cf. Table II) from two molecules of nucleoside diphosphates. If this is true, the amount of nucleoside diphosphates formed by breakdown would be even higher than the amount recorded by the analysis of the final products of degradation.

Breakdown in E. coli *MRE 600*

It was concluded earlier that ribonuclease I probably did not take part in the formation of final products of degradation since neither nucleoside 2′,3′-cyclic phosphates, nor nucleoside 3′-phosphates have been detected at any stage of the process[2]. To test whether ribonucelase I plays a role in the early stages of breakdown, we examined the ability a strain lacking ribonuclease I activity, *E. coli* MRE 600, to break down its RNA under the influence of irradiation. The results (Fig. 4) show exactly the same type and extent of breakdown as in *E. coli* B (cf. Fig. 1). It is obvious that the absence of ribonuclease I does not prevent the strain *E. coli* MRE 600 from breaking down its RNA under the influence of irradiation.

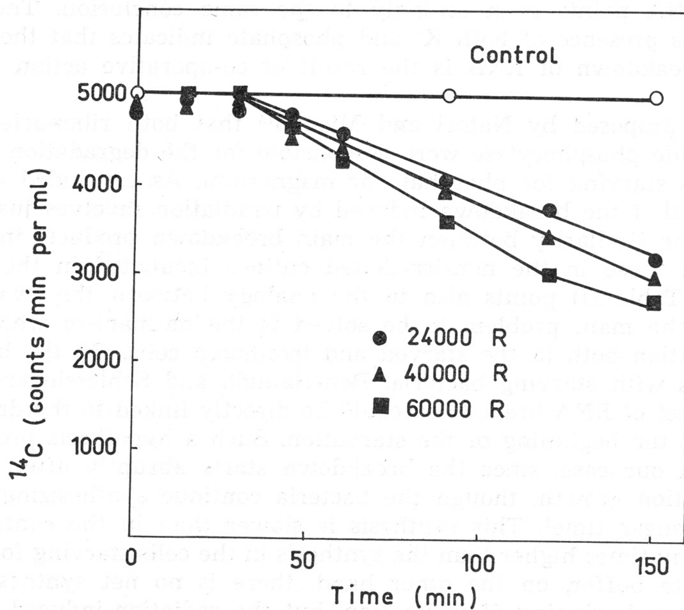

Fig. 4. Breakdown of RNA in *E. coli* MRE 600 irradiated with various doses of X-rays. The bacteria were grown and labelled as described in Fig. 1 for *E. coli* B. Parts of bacterial culture were irradiated with various doses of X-rays, diluted with equal volumes of fresh medium, and incubated at 37°. Radioactivity of RNA was determined in 1 ml. samples.

DISCUSSION

Many attempts have been made to elucidate the role of known enzymes in the breakdown of RNA in *E. coli* starving for some of their nutritional requirements. The conclusion common to all of them is that ribonuclease I is not involved in the degradation, since the mutants of *E. coli* lacking this enzyme were found to degrade their RNA when starved for nitrogen[3], magnesium[15] and phosphorus[15].

Our present experiments with the strain lacking ribonuclease I show that this enzyme is neither involved in any stage of the breakdown of RNA in X-irradiated *E. coli*. The enzyme(s) responsible for this breakdown must be synthesized before irradiation, as shown by the failure of chloramphenicol to influence the rate of degradation. Pre-existing enzymes are also responsible for the breakdown of RNA in *E. coli* starved of nitrogen[3].

Our results regarding the roles of other two known enzymes which can degrade RNA in irradiated *E. coli* suggest that both ribonuclease II and polynucleotide phosphorylase are involved in the process. The action of ribonuclease II is indicated by occurrence of the intermediate breakdown products of high molecular weight[2]. These are known to be produced when ribonuclease II acts on ribosomal RNA[16,17]. Further indication for the action of ribonuclease II is the requirement for K^+ in the incubation medium. Since the optimum concentration of K^+ for the action of the enzyme *in vitro* is close to 0.1 *M* (ref. 16), it is not unexpected that the external supply of the cation is needed to keep intracellular concentration at sufficiently high level. The requirement for phosphate suggests the involvement of polynucleotide phosphorylase. The prominence of UDP and CDP in the products of breakdown of pyrimidine-labelled RNA points even strongly to the same conclusion. The need for simultaneous presence of both K^+ and phosphate indicates that the radiation-induced breakdown of RNA is the result of co-operative action of the two enzymes.

It was proposed by Natori and Mizuno[15] that both ribonuclease II and polynucleotide phosphorylase were responsible for the degradation of RNA in *E. coli* cells starving for phosphate or magnesium. As concluded above, it is very likely that the breakdown induced by irradiation involves just the same enzymes. The similarity between the main breakdown products in irradiated culture and those in the non-irradiated culture incubated in the phosphate buffer (cf. Table II) points also to the analogy between the two processes. Therefore, the main problem to be solved is the mechanism which controls the degradation both in the starved and irradiated cells. On the basis of the experiments with starving bacteria, Ben-Hamida and Schlessinger[3] suggested that the onset of RNA breakdown could be directly linked to the drop in RNA synthesis at the beginning of the starvation. Such a hypothesis probably does not hold in our case, since the breakdown starts abruptly after 30 min. of post-irradiation growth, though the bacteria continue synthesizing new RNA for much longer time[2]. This synthesis is slower than in the control culture, yet it is many times higher than the synthesis in the cells starving for nitrogen[3]. In phosphate buffer, on the other hand, there is no net synthesis of RNA from the very beginning of incubation, but the radiation-induced breakdown of RNA starts again after a lag of about 30 min. As already shown[2] the duration of the lag is independent of the dose of irradiation. Our recent results, which will be published in more details elsewhere[18], show that the same doses of

irradiation result in a very similar rate of breakdown in a number of bacterial strains of very different radiosensitivity. The breakdown of RNA is, therefore, more directly connected with the amount of damage suffered by the cells, than with the fact whether the cell will or will not survive the irradiation. As yet we are unable to propose a mechanism by which the damage caused by irradiation induces the breakdown of RNA, but we believe that the investigation of this mechanism could contribute to the general knowledge about the regulation of the breakdown of RNA in *E. coli.*

Acknowledgment. The authors are pleased to acknowledge the skillful technical assistance of Miss J. Hrženjak. This investigation was supported in part by grants from the Yugoslav Federal Research Funds and Yugoslav Atomic Energy Commission.

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IZVOD

Vjerojatna uloga poznatih enzima u razgradnji RNA kod bakterije *Echerichia coli* nakon zračenja

I. Pečevsky i Ž. Kućan

Razgradnja RNA, obilježene ^{14}C-adeninom, teče kod bakterije *Escherichia coli* ozračene X-zrakama jednakom brzinom u prisutnosti kloramfenikola i bez njega. To ukazuje da razgradnju kataliziraju enzimi sintetizirani prije zračenja. Premda se znatna količina RNA razgrađuje prilikom inkubacije bakterija u nekim otopinama soli, ta je razgradnja pojačana zračenjem samo onda ako inkubacioni medij sadrži i K^+ i fosfate. Među razgradnim produktima RNA, obilježene ^{14}C-adeninom ili ^{14}C--uracilom, nađeni su difosfati nukleozida uz veće količine odgovarajućih monofosfata i nešto trifosfata. Razgradnja RNA kod soja *E. coli* MRE 600, koji nema ribonukleazu I, odvija se jednakom brzinom kao kod soja *E. coli* B. Na osnovu tih i nekih ranije objavljenih podataka zaključeno je da ribonukleaza I ne sudjeluje u razgradnji, dok su za taj proces odlučne ribonukleaza II i polinukleotid fosforilaza.

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Primljeno 12. travnja 1968.