FUNCTIONAL INACTIVATION AND APPEARANCE OF BREAKS IN RNA CHAINS CAUSED BY GAMMA IRRADIATION OF *ESCHERICHIA COLI* RIBOSOMES

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ABSTRACT 70S ribosomes and 30S ribosomal subunits from *Escherichia coli* MRE 600 were exposed to gamma irradiation at −80°C. Exponential decline of activity with dose was observed when the ability of ribosomes to support the synthesis of polyphenylalanine was assayed. Irradiated ribosomes showed also an increased thermal lability. D_{37} values of 2.2 MR and 4.8 MR, corresponding to radiation-sensitive molecular weights of 3.1×10^5 and 1.4×10^5, were determined for inactivation of 70S ribosomes and 30S subunits, respectively. Zone sedimentation analysis of RNA isolated from irradiated bacteria or 30S ribosomal subunits showed that at average, one chain scission occurs per four hits into ribosomal RNA. From these results it was concluded that the integrity of only a part of ribosomal proteins (the sum of their molecular weights not exceeding 1.4×10^5) could be essential for the function of the 30S subunit in the polymerization of phenylalanine. This amount is smaller if the breaks in the RNA chain inactivate the ribosome.

INTRODUCTION

We have been interested in the question of how much damage the ribosome could suffer without losing its biosynthetic capacity. To answer this question two groups of inactivation agents could be used: (*a*) chemical or enzymatic agents reacting specifically with some ribosomal components or their functional groups, and (*b*) physical agents, causing more or less unspecific damages following the absorption of a known amount of energy. In the latter group, both ionizing irradiation (Kućan, 1966) and ultraviolet light (Kagawa, Fukutome, and Kawade, 1967) have been shown to inactivate *E. coli* ribosomes. In both cases exponential decline of activity with dose was observed and the size of sensitive target was estimated. Assuming that the inactivation of ribosomes by ultraviolet light is solely due to photohydration of uracil residues, and taking into account the inactivation cross section of uracil

residues determined in ordered structures, such as tRNA, Kagawa et al. (1967) concluded that the majority of the pyrimidine bases in ribosome may be photolyzed without concomitant inactivation of the particle. On the other hand, from inactivation data obtained by gamma irradiation of lyophilized *E. coli* ribosomes it was estimated that one hit of gamma irradiation into the 70S ribosomes causes the inactivation (Kućan, 1966). However, more recent measurements[1] have shown that the irradiation of ribosomes in the presence of protectors and/or at low temperatures reveals their considerably higher resistance. It was concluded that the first experiments (Kućan, 1966) were done under the conditions allowing contribution of indirect effect by bound water; this is known to interfere severely with the calculations of the size of sensitive target (Henriksen, 1966).

In this paper we wish to report on the gamma ray inactivation studies of 70S ribosome and 30S ribosomal subunit of *E. coli*, performed under the conditions where only the absorption of energy by irradiation target (direct effect) is believed to take place. The results make possible a calculation of average energy required to annihilate the function of the ribosome in the polymerization of phenylalanine in a cell-free system. These measurements are accompanied by determination of the most prominent physical damage caused by irradiation of ribosomes: the breaks in RNA chains. The results clearly indicate that the integrity of only a small fraction of ribosomal components is essential for the function of ribosomes in polyphenylalanine synthesis.

MATERIALS AND METHODS

Bacteria

E. coli MRE 600, used throughout this study, was kindly provided by Dr. H. E. Wade, Microbiological Research Establishment, Porton, England. The bacteria were grown in a medium containing 1% yeast extract, 1% glucose and 0.25 M potassium phosphate (pH 7.0) with strong aeration at 37°C. For isotope labeling the mineral medium was used (Pečevsky and Kućan, 1967); it was supplemented with 50 μCi of adenine-^{14}C (Schwarz Bio Research, Orangeburg, N. Y.; specific activity 53.5 mCi/mmole) per liter of medium during the last 90 min of bacterial growth. Cells were harvested in the late exponential phase, washed with 10 mM magnesium acetate, 10 mM Tris·HCl (pH 7.4), 60 mM NH_4Cl, 6 mM β-mercaptoethanol (standard buffer), and frozen.

Preparation of Ribosomes and Enzyme Fraction

All manipulations were carried out at 4°C. The cells were ground with alumina, and extracted with standard buffer. After the first centrifugation at 30,000 *g* for 20 min, deoxyribonuclease was added to a concentration of 5 μg/ml; the centrifugation was then repeated. Ribosomes were collected by centrifugation at 100,000 *g* for 150 min; the pellet was resuspended in 10 mM magnesium acetate, 10 mM Tris·HCl, pH 7.4, 0.2 M NH_4Cl, and then clarified by centrifugation at 20,000 *g* for 20 min. This centrifugation cycle was repeated twice more using 10 mM Tris·HCl, pH 7.4, 10 mM magnesium acetate ("high-Mg-buffer"). Alternatively, the ribo-

[1] Petranović, D., I. Pečevsky-Kućan, and Ž. Kućan. In preparation.

somes obtained by the first high speed centrifugation were suspended and dialyzed overnight against three changes of 0.1 mM magnesium acetate, 10 mM Tris·HCl, pH 7.4 ("low-Mg-buffer"). The ribosomal subunits were separated by zone sedimentation in a 5–20% linear sucrose density gradient, prepared in the low-Mg-buffer, at 34,000 rpm for 150 min, using a Spinco SW 39 rotor (Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif.). Two-drop fractions were collected; the position of subunits in each gradient was determined by radioactivity measurements in 10 μl samples of all fractions, or by OD 260 measurements in 15 μl samples diluted to 1 ml with water. Appropriate fractions from 3–6 gradients, selected so as to minimize cross-contamination of subunits, were pooled, and freed of sucrose by overnight dialysis against the low-Mg-buffer.

The upper three-quarters of the supernatant fraction, obtained by the first 100,000 *g* centrifugation, were used as a source of enzymes. The fraction was dialyzed overnight against three changes of the standard buffer. The centrifugation at 100,000 *g* for 150 min was then repeated; the upper two-thirds of the supernatant fraction was frozen in small batches and used as needed.

Irradiation

Ribosomes were irradiated from a 3000 Ci^{60} Co gamma ray source at a dose rate of 3760 R/min. 0.1 to 0.2 ml samples were sealed in small test tubes and immersed in dry ice–acetone mixture during and after irradiation. Concentration of 70S ribosomes in the samples was 105 OD 260 units/ml of high-Mg-buffer. 30S ribosomal subunits were irradiated at the concentration of 10 OD 260 units/ml of low-Mg-buffer; in the case of adenine-^{14}C–labeled subunits this concentration corresponded to about 200,000 cpm/ml.

For irradiation of whole cells the exponentially growing culture was used; bacteria were collected by centrifugation and resuspended in 0.01 M Tris·HCl (pH 7.4) to a concentration of 10^{10} cells/ml. 1 ml portions of this suspension were placed in small test tubes and centrifuged. The buffer was drained off, the test tubes sealed, immersed in dry ice–acetone mixture, and irradiated as above.

Assay of the Activity of Ribosomes

Ribosomes were assayed for their ability to support the polyuridylic acid-directed (poly-U) polymerization of phenylalanine; 0.25 ml samples of the reaction mixture contained (in μmole): 15 Tris·HCl, pH 7.4; 10 NH_4Cl; 4 magnesium acetate; 0.75 adenosine triphosphate (ATP); 0.075 guanine triphosphate (GTP); 2.5 phosphoenolpyruvate; 1.5 β-mercaptoethanol; 0.00625 phenylalanine-^{14}C (10 mCi/mmole, Schwarz Bio Research); 5 μg pyruvate kinase (Calbiochem, Los Angeles, Calif.); 0.15 mg tRNA; 20 μg poly-U (Miles Laboratories, Elkhart, Ind.); 0.5–1 mg of protein in 100,000 *g* supernatant fraction; up to 5 OD 260 units of 70S ribosomes. In the assay of 30S ribosomal subunits they were used at the concentration up to 0.3 OD 260 units per sample; the specific activity of phenylalanine-^{14}C was 20 mCi/mmole, and the mixture was supplemented with 0.6 OD 260 units 50S ribosomal subunits. The mixture was incubated for 30 min at 30°C before 2.5 ml. of 5% trichloroacetic acid were added. Samples were heated at 90°C for 15 min, precipitates collected on Millipore filters (Millipore Filter Corp., Bedford, Mass.), washed, dried, and counted in a gas flow counter.

Thermal Inactivation

The suspension of ribosomes, irradiated at −80°C with various doses at a concentration of 105 OD 260 units/ml, was thawed and diluted to 33 OD 260 units/ml in the high-Mg-buffer.

50 μl portions of this suspension were incubated at various temperatures (controlled to ±0.02°C) for 5 min, and then quickly cooled in an ice-water mixture. When all samples were collected, they were supplemented with other components for the synthesis of polyphenylalanine, and assayed as described above.

Preparation of RNA

Total RNA was isolated by a modified phenol method (Pečevsky and Kućan, 1967). The bacteria were ground with quartz sand in the presence of bentonite and 0.2% sodium dodecyl sulfate. Ribonucleoprotein was extracted with 0.1 M NaCl, 0.01 M sodium acetate (pH 5.2), and immediately deproteinized with an equal volume of freshly distilled phenol, saturated with the above buffer. The upper layer obtained by 20 min centrifugation at 12,000 *g* contained 75 ±5% of the original adenine-^{14}C incorporated into bacteria.

RNA from 30S ribosomal subunits was isolated by an analogous procedure omitting, of course, the use of quartz sand. Shaking with phenol was performed at room temperature for 5 min. The recovery of radioactivity in RNA fraction was 80 ±4%.

In order to reveal all strand breaks in RNA, the preparations of RNA were denatured by heating for 4 min at 80°C, and then quickly cooled (Marcot-Queiroz and Monier, 1965).

Sedimentation Analysis of RNA

Linear sucrose density gradients (2.5–15%) were prepared in 0.1 M NaCl, 0.01 M sodium acetate, pH 5.2. 0.1 or 0.2 ml samples (containing about 100,000 cpm of total RNA or 40,000 cpm of RNA from 30S subunits) were layered on the top of gradients and centrifuged at 34,000 *g* for 5 hr in a Spinco rotor SW 39. Two-drop fractions were collected; 0.25 mg of yeast RNA was added to each fraction, followed by precipitation with cold 5% trichloroacetic acid. Precipitates were collected on Millipore filters, dried, and counted in a gas flow counter.

Quantitative Interpretation of Sedimentation Profiles

The molecular weight of RNA is roughly proportional to the square of its sedimentation velocity (Kurland, 1960). Thus, from the analysis of the statistical distribution of the chain lengths (or mass) as the function of the received dose, and from its comparison to the sedimentation profiles, it is possible to determine the average number of breaks per molecule, hence the D_{37}.[2]

We suppose random distribution of breaks over the entire population of RNA molecules, each of length L and mass M_0. The breaks are distributed first, over the molecules, second, within the molecules. The distribution of breaks over the molecules is determined by Poisson's distribution:

$$f_n^m = \frac{m^n \cdot e^{-m}}{n!}, \qquad (1)$$

where f_n^m is the probability of finding n breaks in one RNA molecule, and m denotes the average number of breaks per molecule. We may classify all RNA molecules into groups according to the number of breaks introduced by irradiation, and then calculate statistical distribution of breaks (or chain lengths) within each group.

[2] Charlesby (1954) has treated the random breakage of linear polymers, assuming that only the weight average of the degraded product can be measured. In the present case the complete distribution is known for any received dose and a different approach can be utilized.

Let $g_n(x)$ represent the probability of finding molecular fragments of length x (x is a fraction of the total length L), corresponding to mass M, from the group of RNA molecules broken n times. All the unbroken molecules are of the same size; thus they can be represented by the δ-function:

$$g_0(x) \sim \delta(x - 1).$$

From the group of the molecules broken once, all the molecular fragments in the range $0 < x < 1$ are equally probable:

$$g_1(x) \sim \text{constant}.$$

When $g_1(x)$ is known, $g_2(x)$ can be calculated by assuming one more break per molecule. Quite generally,

$$g_n(x) \sim \int_x^1 g_{n-1}(x') \cdot dx'/x'. \tag{2}$$

Here we denote the integration variable by x' in order to avoid confusion. Integration is taken over all molecular fragments longer than x. From formula 2 it is possible to determine all functions $g_n(x)$:

$$g_2(x) \sim \log x,$$

$$g_3(x) \sim (\log x)^2,$$

$$g_4(x) \sim (\log x)^3,$$

$$\cdots\cdots\cdots\cdots\cdots\cdots$$

$$g_n(x) \sim (\log x)^{n-1}.$$

Experimentally the mass distribution rather than the distribution of the number of molecules is observed. Thus the functions $h_n(x) \sim x \cdot g_n(x)$ correspond to the measured quantity.

The total mass distribution function is

$$W^m(M) \sim \sum_n f_n^m \cdot h_n(x). \tag{3}$$

$h_n(x)$ are the following normalized functions;

$$x \cdot (x - 1),$$

$$2x,$$

$$-4x \cdot \log x,$$

$$4x \cdot (\log x)^2,$$

$$-(8/3)x \cdot (\log x)^3,$$

$$(4/3)x \cdot (\log x)^4,$$

$$\cdots\cdots\cdots\cdots\cdots\cdots$$

The mass distribution depends only upon the average number of breaks per molecule m.

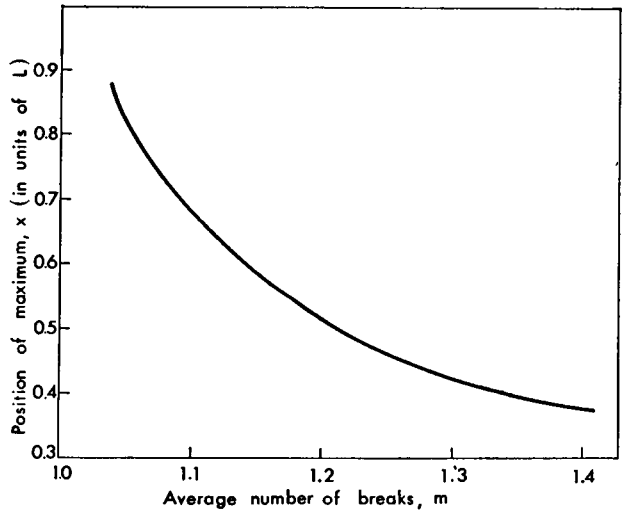

FIGURE 1 The position of the maximum in the sedimentation curves, x (in units of L), as a function of the average number of breaks per molecule, m, as calculated from the mass distribution function $W^m(M)$.

The comparison of the observed sedimentation profile with the function $W^m(x)$ is possible quite generally. However, the comparison is rather simple for certain ranges of m values.

(*a*) For small m, $W^m(x)$ can be well approximated with $f_0^m \cdot h_0(x) + f_1^m \cdot h_1(x)$. Higher terms are small and can be neglected. $h_0(x)$ represents the undamaged molecules. Experimentally it is not the δ-function. Its actual shape is given by the sedimentation profile of the unirradiated molecules. $h_1(x)$ represents the molecules which are broken once. This group of molecules is well represented by the theoretical $h_1(x)$ curve, except at $x \approx 1$. The weight factors f_0^m and f_1^m, and hence m, are determined from the best fit of the two-term sum to the experimental sedimentation curves. The fitting is not attained at $x \approx 1$.

(*b*) The distribution function $W^m(M)$ has a maximum at $x = 1$ (i.e., $M = M_0$). For $m > 1$ there exists an additional maximum, the position of which depends upon m. The correlation between the position of the maximum, x, and the average number of breaks, m, is given in Fig. 1. The sedimentation curve is decomposed first into $h_0(x)$ and the rest, and then m is determined from the position of the maximum of the rest. This way of determination of the average number of breaks is suitable in the range $1 < m < 1.5$.

For 16S RNA both of these approximations were used, whereas 23S RNA was analyzed from the sedimentation profiles of the total ribosomal RNA, for low doses only.

RESULTS

Inactivation of Polyphenylalanine Synthesizing Activity

Preliminary Experiments. To obtain a real measure of the ability of ribosomes to support the polymerization of amino acids, the amount of ^{14}C-labeled amino acid incorporated into acid-precipitable material should be proportional to the concentration of both control and irradiated ribosomes in the assay. Fig. 2 shows that the conditions of the assay fulfill this requirement. Hence, the ratio of

the slope of the line connecting the points obtained with ribosomes irradiated with a certain dose, and the slope obtained with unirradiated ribosomes, represents the measure of activity of irradiated sample. This method, taking three different concentrations of ribosomes irradiated with each dose, was used to construct the inactivation curves shown below.

It is evident that the comparison of initial rates of incorporation reveals the real catalytic capacity of ribosomes (Kućan, 1966). However, it is not clear a priori that the comparison of the amounts of amino acids, polymerized at some later stage of the reaction, would yield the same result. To clear this point, kinetics of the polymerization of phenylalanine was measured with unirradiated as well as with heavily irradiated ribosomes (Fig. 3). The ratio of radioactivity incorporated by irradiated and unirradiated ribosomes remained constant (0.45 $\pm$0.02) throughout the experiment. It follows that longer incubation times are equally suitable for the determination of the activity of ribosomes, and 30 min incubation was chosen as a standard incubation time.

Inactivation Curves for 70S Ribosome and 30S Ribosomal Subunit. The 70S ribosomes were exposed to various doses of gamma irradiation, and assayed for their ability to support the synthesis of polyphenylalanine. The remaining activities, shown in Fig. 4 on a semilogarithmic diagram, follow single hit kinetics. Due to the irradiation conditions used in the experiment (low temperature of irradiation) the slope of the curve is not as steep as that obtained in earlier experiments (Kućan,

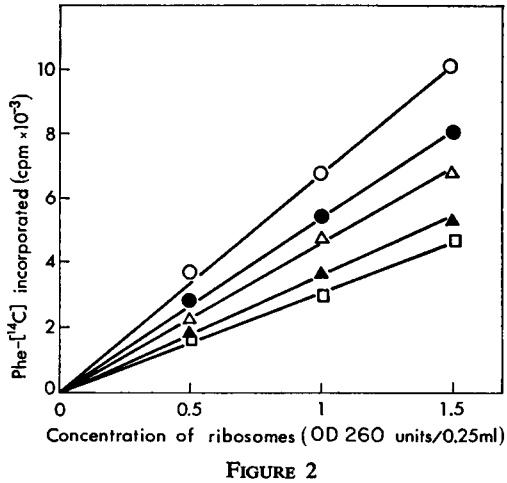

FIGURE 2

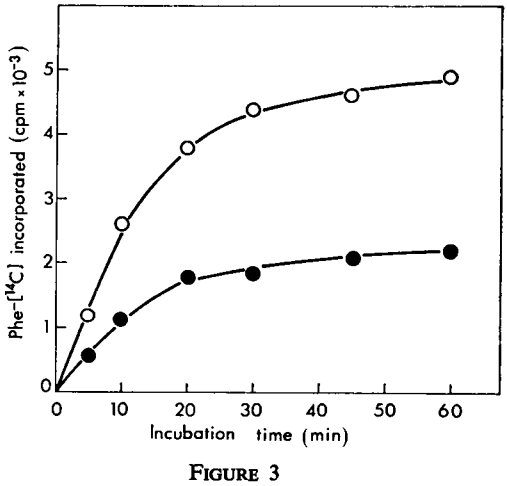

FIGURE 3

FIGURE 2 Incorporation of phenylalanine-^{14}C as a function of the concentration of ribosomes in the assay. Unirradiated ribosomes (open circles) and ribosomes irradiated with 0.37 MR (closed circles), 0.75 MR (open triangles), 1.1 MR (closed triangles), and 1.5 MR (squares), were incubated as described under Materials and Methods.

FIGURE 3 Kinetics of incorporation of phenylalanine-^{14}C in cell-free systems containing unirradiated (open circles) and heavily irradiated ribosomes (closed circles) at the concentration of 2.5 OD 260 units/0.25 ml.

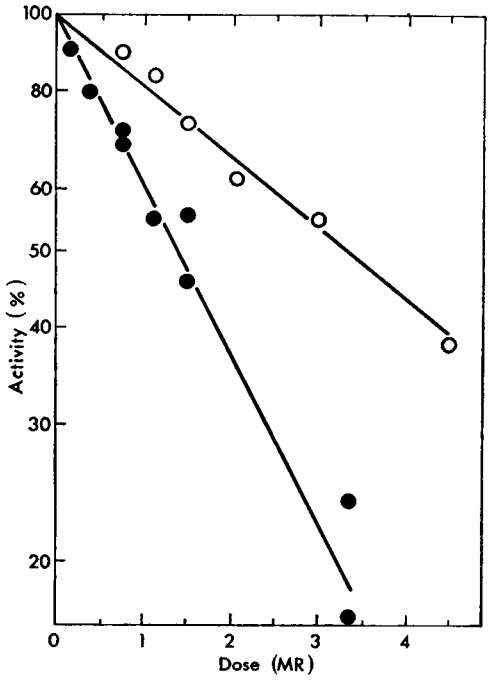

FIGURE 4 Inactivation of 70S ribosomes (closed circles) and 30S ribosomal subunits (open circles) by gamma irradiation. Ribosomes were assayed for their ability to support the polymerization of phenylalanine; an excess of 50S subunits was used in the assay of 30S subunits. Each point is based on the incorporation with three different concentrations of the assayed particles using in part the values from the experiment shown in Fig. 2.

1966). Duplications of the experiment shown in Fig. 4 revealed the D_{37} value for 70S ribosomes of 2.2 ±0.3 MR. Fig. 4 also shows that the exponential decline of activity was obtained when 30S ribosomal subunit was irradiated and assayed in the presence of an excess of 50S subunits. As expected for the smaller target, the 30S ribosomal subunit showed higher resistance to gamma irradiation, and D_{37} of 4.8 ±0.6 MR was estimated.

Thermal Lability of Irradiated Ribosomes. Calculation of number of "hits" of gamma irradiation, needed to annihilate the function of the ribosome (see Discussion), shows that a number of hits into the ribosome is not lethal. Nevertheless, some of these nonlethal hits influence the ribosome; this is indicated by the increased thermal lability of irradiated 70S ribosomes (Fig. 5). While unirradiated ribosomes retain full activity up to 50°C, and lose 50% of their activity by heating for 5 min at 56.4°C, the midpoints of thermal inactivation for ribosomes irradiated with 0.4, 1.5, and 3.3 MR are 55.2, 54, and 52.3°C, respectively. The shape of the inactivation curves of the ribosomes irradiated with higher doses (1.5 and 3.3 MR) clearly points to an increasing fraction of very labile ribosomes in the surviving population.

Breaks in RNA Chains Caused by Irradiation of Ribosomes

Among various radiation-induced physical and chemical changes in the components of ribosomes, those in RNA moiety are, due to the size of this target, expected to

occur at the highest rate. Since the integrity of ribosomal RNA could be of importance in maintaining the proper structure of ribosome, we focused our attention on the appearance of RNA chain scissions in irradiated ribosomes. Both main components, 23S and 16S RNA, were studied; however, the experimental method depended very much on the reproducibility of obtaining unbroken RNA from the unirradiated samples. While 30S ribosomal subunit from *E. coli* MRE 600 yielded in our hands unbroken 16S RNA preparation, the same was not always true for the purified 50S subunit, or even purified 70S ribosomes. Breaks in 23S RNA were especially detectable after thermal denaturation; the action of RNase IV, present in the ribosomes of this strain (Spahr and Gesteland, 1968), might be a probable cause of these breaks. For all these reasons, we studied the breaks in 23S RNA by irradiating the whole cells, extracting the total RNA, and analyzing it by ultracentrifugation, while the occurrence of breaks in 16S RNA was studied by irradiation of purified 30S subunits.

Analysis of Total RNA from Irradiated Bacteria. Our systematic study of the inactivation of ribosomes by gamma irradiation under various conditions[1] showed that the effect, measured after the irradiation at −80°C, cannot be significantly lowered by protection and/or by removal of water. Hence, the irradiation of ribosomes in the environment of other cellular components should not, at this temperature, yield effects different from those obtained by irradiation of purified ribosomes.

Sedimentation profiles of native RNA isolated from irradiated bacteria show, when compared with unirradiated controls, some loss of the material from the 23S peak, as well as some accumulation of the material sedimenting between 4S and

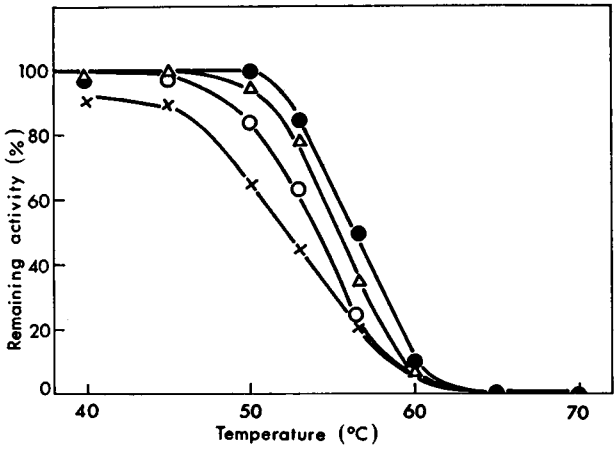

FIGURE 5 Thermal lability of unirradiated ribosomes (closed circles) and ribosomes irradiated with 0.4 MR (triangles), 1.5 MR (open circles), and 3.3 MR (crosses). Samples of ribosomes were incubated for 5 min at indicated temperatures, and then assayed for the incorporation of phenylalanine. 100% incorporation refers to the samples kept at 0°C; its actual value was dose dependent (cf. Fig. 4).

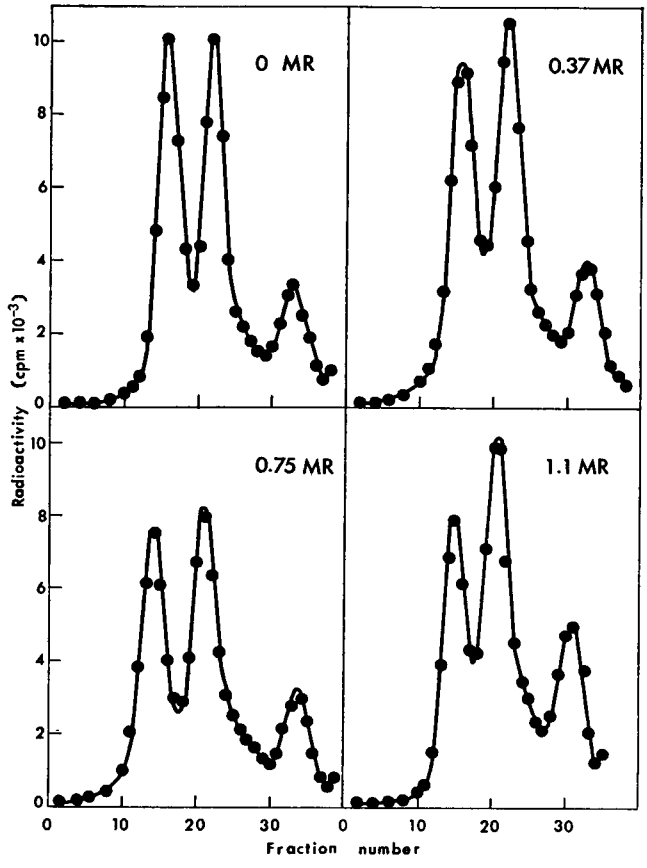

FIGURE 6 Zone sedimentation pattern of RNA isolated from adenine-^{14}C-labeled cells of *E. coli* MRE 600 irradiated with various doses of gamma irradiation. The sedimentation is from right to left.

16S. However, if the RNA samples are denatured prior to sedimentation for the purpose of detecting chain breaks within ordered secondary structures, there is a more pronounced change in sedimentation profiles of irradiated samples (Fig. 6). Decrease of radioactivity of 23S fraction, and accumulation of slower sedimenting material can be seen easily. The loss of radioactivity from the 23S peak in the analysis of denatured samples enables the calculation of D_{37} for the occurrence of chain scissions in this component. The distribution of material around the position of 16S peak in these profiles is too complex, since in this region we find both the accumulation of material, originating from 23S peak, and the concomitant loss of material from both this fraction and the original 16S peak. Hence, these profiles are not suitable for the analysis of breaks in 16S RNA.

Analysis of RNA from Irradiated 30S Subunits. Higher doses of gamma irradiation are needed to cause noticeable changes in the sedimentation profiles of

RNA isolated from 30S ribosomal subunits. Native samples showed again slower changes in sedimentation properties than denatured ones; the latter are shown in Fig. 7 for a number of doses. Two features should be noted in the figure: (*a*) decrease of the 16S peak, detectable already at rather low doses, and (*b*) a shift in the

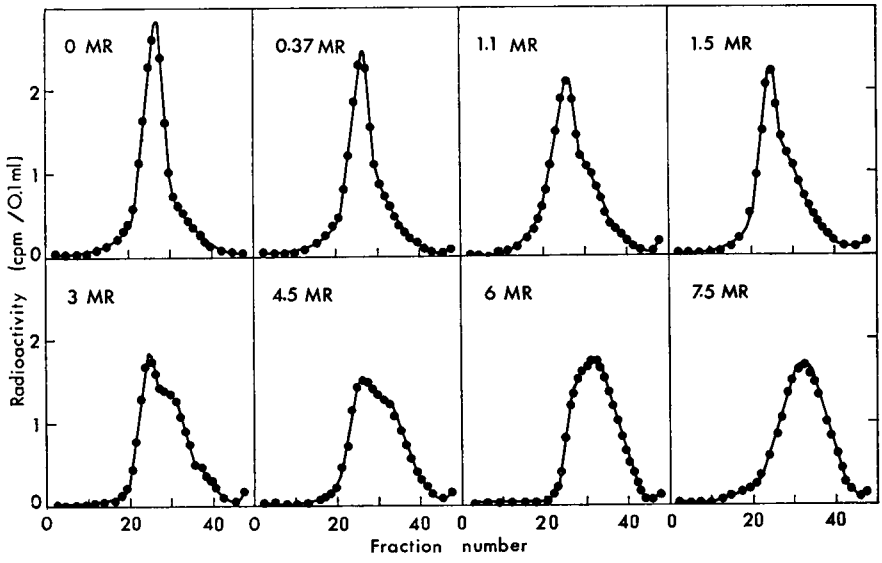

FIGURE 7 Zone sedimentation pattern of RNA isolated from 30S ribosomal subunits irradiated with various doses of gamma irradiation. The sedimentation is from right to left.

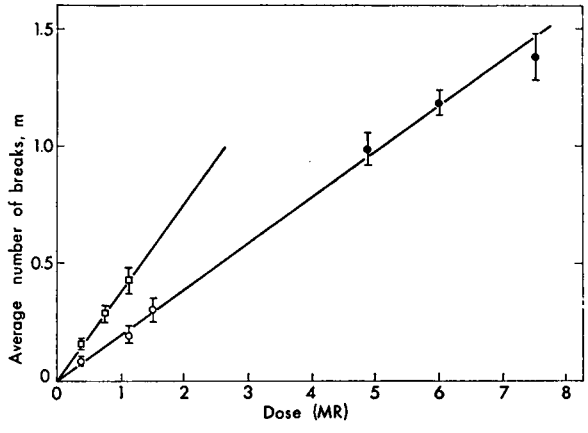

FIGURE 8 Average number of breaks per RNA molecule, m, as a function of the dose of gamma irradiation. The values are obtained by comparison of the observed sedimentation profiles with the theoretical curves $W^m(M)$. Method (*a*) described under Materials and Methods was used for 23S RNA (squares) and 16S RNA at smaller doses (open circles) while method (*b*) was used for 16S RNA at higher doses of irradiation (closed circles).

position of the main peak towards lower S values, obtained with higher doses of irradiation. These two features enable the calculation of D_{37} for 16S RNA.

Determination of D_{37} for 23S and 16S RNA. From the sedimentation curves like those shown in Figs. 6 and 7, and from the analyses outlined above, the average number of breaks was determined for each received dose. Fig. 8 shows the plot of the average number of breaks vs. the dose for both 23S and 16S RNA. Such a plot should be a straight line going through the origin. D_{37} is the dose for $m = 1$. From Fig. 8 we estimate $D_{37} = 2.7 \pm 0.4$ MR and 5.1 ± 0.3 MR for 23S and 16S RNA, respectively. A value for the latter, approximately twice as large, is in good agreement with the molecular weight ratio of the two RNA species.

DISCUSSION

The Size of the Components Essential for the Function of Ribosome

The energy of irradiation is absorbed in a quantized manner. The average energy of a cluster of one or more ionizations ("primary ionization") is about 60 ev in solids of composition similar to that of proteins and nucleic acids (Rauth and Simpson, 1964). From this figure, and from the estimated absorption coefficient of tissue material of 78 ergs/g per R (Andrews, 1961), it can be calculated that 1 R of radiation energy causes 9×10^{11} hits per gram of the target material. Thus, the size of the radiation-sensitive target, expressed as "radiation-sensitive molecular weight" (RSMW) is given by the simple relationship:

$$\text{RSMW} = \frac{6.03 \times 10^{23}}{D_{37} \times 9 \times 10^{11}}, \qquad (4)$$

where D_{37} is the dose (in roentgens) leaving 37% activity, i.e., causing at average 1 hit per target. This relationship has been used very successfully by Ginoza (1967) in computing RSMW for a number of viruses. From D_{37} values, obtained in this paper for the functional inactivation of 70S ribosomes and 30S ribosomal subunits, we calculate RSMW values of $(3.1 \pm 0.4) \times 10^5$ and $(1.4 \pm 0.2) \times 10^5$, respectively. These values are almost one order of magnitude lower than the actual molecular weights of ribosomes, and some three to four times lower than the molecular weights of their largest component, RNA. Since there are good reasons to suppose that the absorption of energy of gamma radiation in such a large target is random in space, the above calculation shows that the energy of 60 ev can be absorbed by about 90% of the total volume of 70S ribosome or 30S ribosomal subunit without impairing the biological function of these particles. (The result does not mean, of course, that the accumulation of 10 hits per ribosome is needed for inactivations: in such a case nonexponential survival curves would have been obtained). The resistance of ribosomes becomes apparent when their radiosensitivity is compared to that of other macromolecular targets. A good agreement between RSMW and

actual molecular weight has been obtained for enzymes (cf. Hutchinson and Pollard, 1961); viruses containing single-stranded DNA or RNA also show an excellent agreement between RSMW and the molecular weight of the infective component (Ginoza, 1967). There are no reasons why the basic molecular mechanisms, following the absorption of 60 ev by ribosomal components, should be different from the corresponding mechanisms in other proteins and in viral RNA. Hence, the conclusion follows that the majority of ribosomal proteins, as well as the majority of ribosomal RNA, have no functional role in the polymerization of phenylalanine. If we ascribe all functions of ribosome to its protein moiety, and if we take 15,000 as the average molecular weight of ribosomal proteins (Moore, Traut, Noller, Pearson, and Delius, 1968), then the calculated RSMW values of 3.1×10^5 and 1.4×10^5 indicate that only up to 20 proteins in the 70S ribosome and up to 10 proteins in the 30S subunit are essential for the function. (If the inactivation of 70S ribosome reflects the radiosensitivity of the 50S component, what is not excluded by our experiments, then the first value applies actually to this subunit). These are in fact maximum numbers; actual values are lower if some of the hits into ribosomal RNA also annihilate the function of the particle (see the second part of Discussion). To determine whether the rest of ribosomal proteins have only a structural and not a functional role, or their function is limited to the steps not assayed in the poly-U–phenylalanine system (such as chain initiation and termination), remains the task of future experiments.

Strictly exponential inactivation curves, obtained under the direct effect conditions (Fig. 4), and also by irradiation of lyophilized ribosomes or ribosomal suspensions (Kućan, 1966), indicate that the events within a well defined volume cause inactivation. Thermal lability of the ribosomes surviving irradiation, which increases with the dose, suggests that a fraction of hits, which do not cause immediate inactivation of the ribosome, is in fact potentially lethal. A possible explanation is that some hits weaken the structure of ribosomes by breaking bonds not involved in the functional sites; however, at increased temperatures, the over-all structure of ribosome changes to a nonfunctional state.

Breaks in RNA Chains and the Function of Ribosome

The above calculations have shown that the sensitive target of a ribosome is considerably smaller than its RNA; hence, the absorption of the energy of primary ionization by RNA need not result in the inactivation of the function of ribosome. Since covalent changes are produced as the consequence of primary ionization, it may be concluded that the ribosomes with chemically changed (but not necessarily broken) RNA are still active. This is in good agreement with the conclusion of Kagawa et al. (1967) that the ribosomes with photochemically changed RNA may remain active in protein synthesis, and also with the finding of Nomura, Traub, and Bechman (1968) that changed (but not broken) ribosomal RNA can be used for

reconstitution of active 30S ribosomal subunits. However, the question arises whether the ribosomes with broken RNA can or cannot function in protein synthesis.

From D_{37} values for the chain scission in 23S and 16S RNA, using the equation 4, we obtain RSMW values of 2.6×10^5 and 1.4×10^5, respectively. By comparing these values with the actual molecular weights of the two kinds of RNA molecules, we conclude that only about 25% of the hits into ribosomal RNA result in the breaks of RNA chain. This value is very low if compared to the efficiency of 75% for the single strand breaks in double-stranded viral DNA (Ginoza, 1967; Taylor and Ginoza, 1967), but is identical to the efficiency of breaking single-stranded DNA (Lytle and Ginoza, 1969) and reasonably close to the early estimate of 40% for the appearance of breaks in the RNA of tobacco mosaic virus (Englander, Buzzell, and Lauffer, 1960).

Let us now compare the appearance of breaks in 16S RNA and the functional inactivation of the 30S subunit. For both events the same RSMW (1.4×10^5) was obtained. From this coincidence two possibilities arise:

(*a*) The sensitive target for inactivation is exclusively within RNA moiety; hence, only the ribosomes with breaks in RNA chain are inactive in protein synthesis, while protein components are not essential for the function.

(*b*) The sensitive target for inactivation is partially or totally situated in the protein moiety; some of the ribosomal proteins, up to the sum of molecular weights of 1.4×10^5, are essential for the functioning of the 30S ribosomal subunit.

At the moment it is not possible to decide which of these two possibilities is true, but the developing knowledge about ribosomal proteins might, on the basis of our results, indirectly demonstrate whether the ribosome (or at least the 30S subunit) with broken RNA is still active in protein synthesis. Namely, if some of the protein constituents of the 30S subunit are found to possess specific function in the polymerization of phenylalanine, it will mean that the breaks in a part of 16S RNA may occur without impairing the activity of the ribosome. As an extreme, if proteins with a sum of molecular weights of 1.4×10^5 are found to possess specific functions in the polymerization reaction, a conclusion will follow that no breaks whatsoever in 16S RNA are damaging the function. Though there are no direct evidences in favor of any of the two mentioned possibilities, the first one does not seem very likely, since the enzymatic cleavage of RNA by "aging" the ribosomes does not result in parallel loss of their activity in the polymerization of phenylalanine (Szer, 1969), and one may suppose that the breaks caused by irradiation will have the same effect.

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REFERENCES

ANDREWS, H. I. 1961. *In* Radiation Biophysics. Prentice-Hall, Inc., Englewood Cliffs, N. J. 194.

CHARLESBY, A. 1954. *Proc. Roy. Soc.* (*London*). **224A**:120.

ENGLANDER, S. W., A. BUZZELL, and M. A. LAUFFER. 1960. *Biochim. Biophys. Acta.* **40**:385.
GINOZA, W. 1967. *Annu. Rev. Microbiol.* **21**:325.
HENRIKSEN, T. 1966. *Radiat. Res.* **27**:694.
HUTCHINSON, F., and E. C. POLLARD. 1961. *In* Mechanisms in Radiobiology. M. Errera and A. Forssberg, editors. Academic Press, Inc., New York. **1**:71.
KAGAWA, H. H. FUKUTOME, and Y. KAWADE. 1967. *J. Mol. Biol.* **26**:249.
KUĆAN, Ž. 1966. *Radiat. Res.* **27**:229.
KURLAND, C. G. 1960. *J. Mol. Biol.* **2**:83.
LYTLE, C. D., and W. GINOZA. 1969. *Int. J. Radiat. Biol. Related Stud. Phys. Chem. Med.* **14**:553.
MARCOT-QUEIROZ, J., and R. MONIER. 1965. *J. Mol. Biol.* **14**:490.
MOORE, P. B., R. R. TRAUT, H. NOLLER, P. PEARSON, and H. DELIUS. 1968. *J. Mol. Biol.* **31**:441.
NOMURA, M., P. TRAUB, and H. BECHMAN. 1968. *Nature* (*London*). **219**:793.
PEČEVSKY, I., and Ž. KUĆAN. 1967. *Biochim. Biophys. Acta.* **145**:310.
RAUTH, A. M., and J. A. SIMPSON. 1964. *Radiat. Res.* **22**:643.
SPAHR, P. F., and R. F. GESTELAND. 1968. *Proc. Nat. Acad. Sci. U.S.A.* **59**:876.
SZER, W. 1969. *Biochem. Biophys. Res. Commun.* **35**:653.
TAYLOR, W. D., and W. GINOZA. 1967. *Proc. Nat. Acad. Sci. U.S.A.* **58**:1753.