THE EFFECT OF MUTATION ON RIBONUCLEIC ACID, PROTEIN AND RIBONUCLEASE FORMATION IN *NEUROSPORA CRASSA*

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SUMMARY

Electrophoretic and biochemical analyses have been carried out to determine whether gene mutation in *Neurospora crassa* can elicit gross changes in nucleic acid or protein formation. Alterations in ribonucleic acid, ribonuclease and total protein formation which were observed during growth could not be specifically attributed to gene mutation.

The ribonuclease of *N. crassa* resembles ribonucleases from other sources in many of its properties. However, the *Neurospora* enzyme can be completely inhibited by ethylenediaminetetraacetic acid.

INTRODUCTION

That specific gene mutations can cause changes in the properties of specific enzymes is well known. During the past decade, a number of such instances have been noted, and certain of these have been studied in detail[1-3]. All of the cases studied to date, however, relate to minor components of the total protein formed by the organism. As enzyme formation is genetically determined, the question arises—does a gene change resulting in an alteration of a specific enzyme affect only this enzyme, or are there additional macromolecular changes associated with the genetic damage which are not detected by the usual screening methods, *i.e.*, can genetic change give rise to a gross alteration in the protein or nucleic acid composition of a cell? An answer to this problem has been sought by establishing the electrophoretic pattern of extracts of wild type *Neurospora crassa* and comparing this with the pattern found in certain mutants. No changes in protein maxima were observed, but a difference in a ribonucleic acid component was noted. Further experiments indicated that abnormal electrophoretic patterns can result from differences in growth rate but that no major differences in protein and nucleic acid components occur which are specifically attributable to single gene mutation. In the present paper this work will be summarized. In the course of these investigations, however, a good deal of detailed

Abbreviations: RNA, ribonucleic acid; DNA, deoxyribonucleic acid; ATP, adenosine triphosphate; EDTA ethylene diaminetetraaceticacid; TCA, trichloroacetic acid.

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data concerning the electrophoretic characterization of *Neurospora crassa* extracts and the properties of *Neurospora* ribonuclease have been compiled. Not all of the data will be recorded here, and the interested reader is referred to the paper by SUSKIND[4].

MATERIALS AND METHODS

Strains

The cultures were grown either as stationary cultures in 2-l Fernbach flasks containing 500 ml of minimal medium plus 2% sucrose[5], or as aerated cultures grown in 5-gallon carboys. All wild type and morphological mutant cultures were grown on a minimal medium plus 1% glucose or 2% sucrose. Biochemical mutant strains were grown on minimal medium supplemented with the appropriate nutrient. After growth, the mycelia were harvested, rinsed with distilled water and stored at —20°, or the mycelia were immediately lyophilized and the lyophilized mycelia were stored at —20°.

Preparation of extracts for analysis

Extracts suitable for electrophoretic analysis were prepared as follows: 500 mg of lyophilized mycelia were ground to a fine powder with a mortar and pestle. The powder was extracted with 10 ml of cold 0.1 *M* phosphate buffer, at pH 7.5–7.8 by shaking with glass beads in the cold for 1–2 h. The extracts were centrifuged at about 3200 rev./min for 0.5 h. The supernatant solutions were subsequently centrifuged for 60 min in a Spinco Model L Preparative Ultracentrifuge at 55,150 × *g*. The clear supernatant solutions were withdrawn using a needle and syringe.

Nucleic acid and protein determinations

All extracts were either stored at —20° or analyzed immediately for nucleic acid and protein content. Ribonucleic acid was estimated by the SCHNEIDER technic[6] using orcinol and u.v. absorption methods for determining ribose and base respectively. DNA was measured by the DISCHE cysteine–HCl method[7] following the procedure described by STUMPF[8]. Protein was determined by the biuret, micro-Kjeldahl or LOWRY methods[9–11].

Electrophoretic analysis

Dialysis was carried out in an Aminco internal stirring mechanical dialyzer at 2°. Three electrophoretic buffers, covering the pH range 6.9–10.0 were used with satisfactory results: cacodylate buffer, pH 6.9, $\mu = 0.1$; veronal buffer, pH 8.6, $\mu = 0.1$; and ammonia buffer, pH 10.0, $\mu = 0.1$ (see ref. 12–14). An Aminco-Stern electrophoresis unit was employed and the final protein concentration of the extracts examined was ordinarily between 1% and 1.5%.

Determination of ribonuclease activity

Ribonuclease activity was assayed using yeast RNA (Schwarz Laboratories) as substrate, in the presence of 0.1 *M* phosphate buffer, pH 7.5 at 37°. A 2% solution of yeast nucleic acid in distilled water (containing 0.84% acid precipitable nucleic acid), adjusted to pH 6.0, served as the stock from which suitable aliquots were taken. Activity was measured by following the decrease in acid-precipitable nucleic acid

using the SCHNEIDER method[6]. The samples were analyzed for pentose by the orcinol method and/or for base by their absorption at 260 mμ.

A unit of ribonuclease activity is defined as the amount of enzyme which depolymerizes 1.0 mg of RNA in 30 min at 37°.

The dry weights, ribonucleic acid and protein content of the lyophilized mycelia are expressed in mg per pad, while ribonuclease activity is given in units of enzyme activity per pad.

EXPERIMENTAL

Electrophoretic results: wild-type studies

Experiments were carried out employing several wild-type strains to establish the normal electrophoretic pattern of *Neurospora crassa* extracts and to determine the effect of culture and extraction conditions on this pattern.

A constant and characteristic electrophoretic pattern was found for extracts of various wild-type strains grown, prepared and tested under a variety of conditions.

Of the four maxima observed, only the fastest moving acid component was isolated and identified. This component had a mobility approximating that of ribonucleic acid. Employing a sampling apparatus, small amounts of this component were withdrawn from the ascending limb of the electrophoretic cell. Ribonucleic

TABLE I

ELECTROPHORETIC MAXIMUM NO. 1 IN WILD TYPE *N. crassa* EXTRACTS

Mobilities expressed as $\mu \times 10^{-5}$ cm^2/sec^{-1}, V^{-1}.

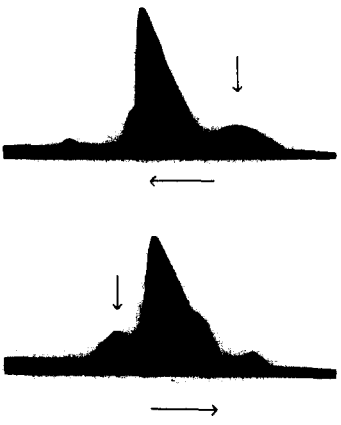
Electrophoretic maximum No.	*Limb*	*Mobility*	*Relative concentration* %	*mg RNA / ml*	*mg protein / ml*	*mg RNA / mg protein*
1	Asc.	—11.2	3.9	0.134	0.038	3.54
	Desc.	— 9.0	4.2	—	—	—
2	Asc.	— 7.1	—	—	—	—
	Desc.	— 5.4	Combined 2, 3 (asc.) 78.0	—	—	—
3	Asc.	— 6.1		—	—	—
	Desc.	— 2.7	(desc.) 76.8	—	—	—
4	Asc.	0	12.1	—	—	—
	Desc.	0	12.8	—	—	—
Total extract (undialyzed)		—	—	2.1	15.5	0.136

acid, DNA and protein determinations were run on the sample and the nucleic acid/protein ratio of the fraction and of the extract were compared. These data together with relative concentration measurements of the maxima are presented in Table I. Component 1, comprising approx. 4% of the total area, consists predominantly of RNA, the RNA/protein ratio of component 1 being 26 times higher than the crude extract value. No DNA was detected in this fraction.

Mutant studies

Several morphological mutants were selected for comparison with the wild-type.

Those mutants were chosen which showed the most striking aberrancies in growth and physical appearance, since it seemed more likely that gross electrophoretic changes might be discernable in this group. Electrophoretic patterns were obtained which indicated a major change in the number of maxima present in extracts of two colonial mutants, Y-9833a and Y-7381 and a microconidial strain, JH-146 (see ref. 15).



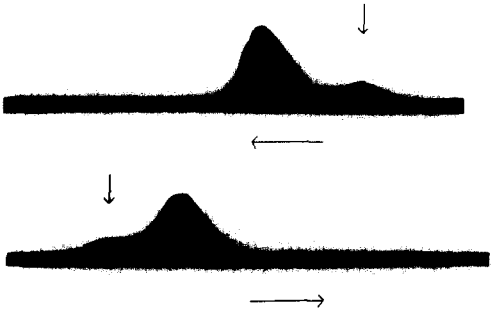


Fig. 2. The electrophoretic pattern of an extract of *N. crassa* colonial mutant, strain Y-7381; 2580 sec migration in 0.1 μ veronal buffer, pH 8.6, at a potential gradient of 8.0 V/cm; 1.1 % total protein.

Fig. 1. The electrophoretic pattern of an extract of wild type *N. crassa*, strain 5265A; 5040 sec migration in 0.1 μ veronal buffer, pH 8.6 at a potential gradient of 5.0 V/cm; 1.5 % total protein.

Extracts of the mutants characteristically lacked the ribonucleic acid-containing component present in the wild-type preparations. This is shown in Fig. 2. A tryptophan-requiring mutant (td_2) and a tryptophan-independent suppressed mutant (td_2su_2) were tested and extracts of these strains exhibited the same pattern noted in the morphological mutants.

The ribonuclease of Neurospora crassa

A substantial decrease in the RNA content of *Neurospora* extracts occurred during extraction and dialysis prior to electrophoresis. This loss was due in part to the action of ribonuclease. Experiments were carried out to characterize the ribonuclease of *N. crassa*, to establish the conditions necessary for inhibition of this activity in the extracts and to determine whether the loss of the RNA component in the mutant extracts represented an RNA difference associated with genetic change, with growth or with an altered or enhanced ribonuclease activity.

Extracts were prepared and assayed for ribonuclease activity as described in the section on MATERIALS AND METHODS. Ultracentrifugation of crude extracts at 41,190 $\times$ g for 90 min, did not sediment ribonuclease activity. No experiments were carried out on the 100,000 $\times$ g fraction which would presumably have contained the ribosomes[16].

An inhibitory effect of ribonucleic acid on *Neurospora* ribonuclease was noted which was similar to the results reported by LAMANNA AND MALLETTE[17] for crystalline pancreatic ribonuclease. The effect of substrate concentration. on activity is shown in Table II. The enzyme is inhibited by the products of RNA depolymerization; however, under the conditions of the assay, activity is linear with time for 30 min.

Attempts to inhibit the enzyme with purine and pyrimidine bases, or with nucleosides and nucleotides, were unsuccessful at concentrations as high as 10^{-2} *M*, with the exception of ATP, which was inhibitory at $8 \cdot 10^{-3}$ *M*.

Maximum activity is found at pH 7.5 with about 75 % loss in activity at pH 6.0. Total inactivation occurs at pH 9.2. An increase in phosphate from 0.2 *M*–0.5 *M*,

TABLE II

THE EFFECT OF RNA CONCENTRATION ON THE ACTIVITY OF *N. crassa* RIBONUCLEASE

Tubes contained 0.2 ml *N. crassa* extract; RNA (20 mg/ml stock solution); 0.1 *M* PO_4 pH 7.5. Total volume per tube equaled 1.2 ml. Incubation was for 30 min at 37°.

mg RNA/tube	*% RNA hydrolyzed*	*mg RNA hydrolyzed*
0.8	76	0.608
1.6	60	0.960
2.4	50	1.20
3.2	42	1.34
4.0	26	1.04
6.0	2	0.01

results in a loss of activity, a 45 % reduction occurring at 0.5 *M*. Maximum activity is found at 60° in 0.1 *M* phosphate buffer. The enzyme is resistant to elevated temperatures for prolonged periods of time which should prove of considerable value in purification.

Dialysis of the enzyme preparation causes no loss in activity, eliminating the likelihood of a readily dialyzable prosthetic group. Almost all of the divalent ions employed at concentrations of 10^{-2} *M* caused inhibition of the enzyme. The most effective inhibitors at 10^{-3} *M* were copper and cobalt. Both ferrous and ferric ions were inhibitory.

The purine analogues benzimadazole and 2-amino benzimadazole[18] were also tested as inhibitors. Benzimadazole at a concentration of 10^{-2} *M* caused no reduction in activity, while the 2-amino derivative was moderately inhibitory. The possibility of inhibiting *Neurospora* ribonuclease activity with EDTA at several pH values was examined. The inhibitory effect of EDTA is most effective at pH 7.0. The chelating action of EDTA is known to be most effective at pH 7.0, which corresponds to the pH dependence of EDTA inhibition.

The fact that EDTA inhibits the action of *Neurospora* ribonuclease was used to prevent the loss of RNA during extraction,dialysis and electrophoretic analysis. As seen in Table III, the presence of $6 \cdot 10^{-3}$ *M* EDTA inhibits the breakdown of Neurospora RNA 57 %. At 0.1 *M* EDTA, no significant ribonuclease activity remains. The inhibitory effect of EDTA can be almost completely reversed by dialysis against 0.05 *M* phosphate buffer, pH 7.8, for 24 h.

The addition of EDTA during dialysis permitted the detection of a small RNA electrophoretic component in the mutant extract. In the absence of EDTA, under identical conditions, there was a 42 % drop in the RNA/protein ratio and no component was visible. It should be emphasized that a comparison of the RNA/protein ratios of undialyzed mutant and wild-type extracts, freshly prepared from cultures of approximately equal age, gave a mutant ratio which was 40–70 % below the

TABLE III

THE EXTRACTION OF *N. crassa* RNA AND PROTEIN
THE EFFECT OF EDTA DURING EXTRACTION ON THE RATE OF DEPOLYMERIZATION OF *Neurospora* RNA

Preparation	*EDTA Ratio*	*Minutes incubated at 37°*					
		10		*20*		*30*	
		mg RNA hydrol.	*% inhibit*	*mg RNA hydrol.*	*% inhibit*	*mg RNA hydrol.*	*% inhibit.*
Control — no EDTA	—	0.790	—	1.20	—	1.48	—
EDTA $6 \cdot 10^{-3}$ *M*	1	0.333	58	0.486	60	0.640	57
EDTA $3 \cdot 10^{-2}$ *M*	5	0.181	77	0.286	76	0.364	75
EDTA $6 \cdot 10^{-2}$ *M*	10	0.082	90	0.166	86	0.166	89
EDTA 0.12 *M*	20	0.02	97	0.02	97	0.02	97

TABLE IV

RNA, PROTEIN AND RIBONUCLEASE FORMATION IN WILD TYPE STRAINS OF *N. crassa*

Strain	*Age (h)*	*mg dry wt./pad*	*mg RNA / pad**	*mg protein / pad**	*mg RNA / mg protein*	*Ribonuclease units/pad**
5256A	36	170	13.1	60.0	0.219	18.3
	48	355	18.1	106.0	0.171	32.6
	96	1030	39.2	252.0	0.155	62.0
	192	1800	64.7	396.0	0.163	172.0
Chilton a	48	410	18.8	93.5	0.201	31.2
	72	900	32.3	173.0	0.187	46.9
	96	1300	40.3	228.0	0.177	41.5
	192	1800	64.7	346.0	0.187	93.5

* 1. mg RNA or protein per pad $= \dfrac{\text{mg RNA or protein/ml of extract}}{\text{mg lyophilized mycelia/ml of extract}} \times$ dry wt. of pad.

2. ribonuclease units per pad = ribonuclease units/ml of extract $\times \dfrac{\text{mg lyophilized mycelia per pad}}{\text{50 mg lyophilized mycelia/ml of extract}}$

TABLE V

RNA, PROTEIN AND RIBONUCLEASE FORMATION IN MUTANT STRAINS OF *N. crassa*

Strain	*Age (h)*	*mg dry wt./pad*	*mg RNA / pad**	*mg protein / pad**	*mg RNA / mg protein*	*Ribonuclease units/pad**
Y-9833a	48	340	12.9	76.5	0.169	13.6
	96	1170	31.6	206.0	0.153	18.8
	192	1260	27.7	212.0	0.131	10.1
C-83a	48	150	7.6	42.0	0.181	9.5
	72	430	17.2	110.0	0.156	27.0
	120	890	39.8	266.0	0.149	63.5
	168	970	43.3	300.0	0.143	74.0
	192	1100	41.0	332.0	0.123	61.0
10575	48	165	5.8	24.4	0.238	8.0
	96	1920	32.6	107.0	0.157	145.0
	192	2120	25.4	186.0	0.137	76.5

* See under Table IV.

wild-type value. Hence, at least two factors play a role in resolving the RNA electrophoretic component in the mutant extracts: the initial RNA/protein ratio of the sample and the extent of RNA depolymerization by ribonuclease during the preparative procedure.

Growth studies

The ribonucleic acid, protein, and ribonuclease concentrations of several wild-type and mutant strains were examined at intervals during an 8-day growth period. A comparison of the data from wild-type strains 5256A and Chilton A is shown in Table IV. The RNA and protein values for the two strains appear to be in good agreement. It can be seen that neither culture has reached maximum growth at 192 h, and that RNA and protein formation appear to be roughly proportional to the rate of growth. It can also be seen from the 36 and 48-h values, that the maximum RNA/protein ratios occur in young cultures. Between 72 and 192 h, this ratio changes very little. The ribonuclease activities of the two strains, although quantitatively different, nevertheless increase with age.

Colonial mutant Y-9833a, and tryptophan mutants td_1[19] and 10575 behaved in an entirely different manner. These data are presented in Table V. It can be seen that the net synthesis of RNA and protein reached a maximum in two of the mutants, Y-9833a and 10575 at about 96 h. In td_1 the RNA value leveled off at about 120 h while protein formation continued. In all of these mutants, the RNA/protein ratio began to decrease significantly once the period of maximum RNA synthesis had been attained. Again, the highest RNA/protein ratios were observed in the young cultures.

The course of ribonuclease formation also differed from the wild-type strains. In the mutants, either ribonuclease activity reached a maximum or sharply decreased concurrent with the decrease in RNA synthesis. The decrease in intracellular ribonuclease activity, which occurred in some cases when the cultures had attained maximum growth, suggested that the enzyme might be released into the culture medium during this period. Aliquots of filtrates of several strains were tested for ribonuclease activity and the extracellular and intracellular enzyme values were compared. As seen in Table VI, the culture filtrates contain considerable ribonuclease activity. This activity in general appears to be maintained at a low level for about 3–5 days and then begins to increase as the culture ages. This increase in extracellular ribonuclease activity has been observed even after maximum RNA formation has been attained. The appearance of extracellular ribonuclease activity is not accompanied by a significant change in the 280 mμ/260 mμ ratio in the filtrate nor by the appearance of TCA precipitable material. Hence, it would appear unlikely that large-scale autolysis had occurred during the course of these growth experiments.

Since these data suggest that certain mutations may affect jointly the course of RNA, protein and ribonuclease formation, it was of considerable importance to determine whether a mutant possessing the ability to grow at wild-type rate would have the wild-type pattern of RNA, protein and ribonuclease formation during an 8-day growth period. An inositolless mutant, WS-7336, exhibited essentially wild-type growth rate when grown on minimal medium plus 10 μg/ml inositol and in this instance ribonucleic acid, protein and ribonuclease formation occurred in a typically wild-type manner. Furthermore, the changes observed in most of the aberrantly growing mutants could be elicited in a non-mutant strain. It is known that colonial-type

TABLE VI

INTRACELLULAR AND EXTRACELLULAR RIBONUCLEASE ACTIVITY IN *N. crassa*

Strain	*Age (h)*	*Ribonuclease units/pad*	*Total filtrate ribonuclease units**	*Extracellular/intracellular activities*
C-83 (td_1)	72	27.0	164.0	6.0
	120	63.5	234.0	3.7
	168	74.0	312.0	4.2
	192	61.0	432.0	7.1
S-1952 (td_2)	72	25.6	247.0	9.1
	120	42.5	650.0	15.5
	168	80.0	1170.0	14.7
	192	96.5	1380.0	14.4
Y-9833a	72	24.3	208.0	8.5
	120	36.5	354.0	9.7
	192	47.0	505.0	11.0

* Total filtrate ribonuclease units = ribonuclease units/ml of filtrate × total volume of filtrate after pads have been harvested.

TABLE VII

THE EFFECT OF SORBOSE ON RNA, PROTEIN AND RIBONUCLEASE FORMATION IN WILD TYPE STRAIN 5256A

Strain	*Age (h)*	*mg dry wt./pad*	*mg RNA / pad*	*mg protein / pad*	*mg RNA / mg protein*	*Ribonuclease units/pad*
5256A	48	105	6.7	34.0	0.197	8.4
	72	450	22.2	139.0	0.159	26.0
	120	570	15.5	125.0	0.124	68.0
	168	750	13.0	127.0	0.102	105.0
	192	770	7.6	92.0	0.083	132.0

growth can be induced in wild-type strains of *N. crassa* by employing suitable ratios of sorbose and sucrose in the medium[20]. Using 1 % sorbose, and 0.5 % sucrose, an altered growth response of strain 5256A was observed. As shown in Table VII, a marked difference was also observed with respect to RNA, protein and ribonuclease formation.

DISCUSSION

The results of growth experiments with *Neurospora* and with other organisms[21–23] show that a high RNA/protein ratio is obtained in actively growing cells, while in old cells the ratio decreases significantly. Consequently a likely explanation for the altered electrophoretic pattern and the low RNA/protein ratio in certain *Neurospora* mutants rests in their modified growth rate. In certain mutant strains, RNA, protein and ribonuclease net synthesis terminate prematurely. This is accompanied by a decrease in the RNA/protein ratio of the extract, and by an increase in extracellular ribonuclease activity.

The low RNA/protein ratios found in older cultures might be accounted for in two ways: either by a diminished rate of RNA synthesis, or by an actual loss of

RNA in the mycelia. As the growth rate decreases there is a concomitant decline in intracellular ribonuclease activity, while the extracellular activity increases. It does not appear that autolysis of the culture can account for these results. It is conceivable that the increase in extracellular ribonuclease activity and the decrease in intracellular RNA may reflect a liberation or activation of the intracellular enzyme at certain sites in the cell, *i.e.*, the ribosomes[16].

The ribonuclease from *Neurospora crassa* appears similar in several respects to ribonucleases from other sources[24]. Activity is inhibited both by high substrate concentration and by the accumulation of the products of RNA depolymerization. No loss of activity occurs as a result of dialysis, and the enzyme is heat-resistant. It appears that the inhibition of *Neurospora crassa* ribonuclease by the chelating agent EDTA is unique. This inhibition is pH dependent and can be reversed by dialyzing the inhibited enzyme. No metal requirement for the enzyme can be demonstrated.

The fact that *Neurospora* ribonuclease activity is heat-resistant, and that it can be obtained from both cells and culture filtrates, would make possible its rapid purification. An extensive purification of this enzyme from *Neurospora* has been recently undertaken by SINGER *et al.*[25] and the sedimentation constant suggests a molecular weight similar to pancreatic ribonuclease.

No evidence was obtained for a qualitative difference between ribonuclease from wild-type and mutant strains, but such a difference might be found by suitable screening procedures. In view of the available information on ribonuclease structure and function[24-27], mutants of this type could prove most informative in studies on gene–enzyme relationships at the molecular level.

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THE NUCLEIC ACID CONTENT OF *ESCHERICHIA COLI* STRAINS B AND B/R

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SUMMARY

The differences in the DNA and RNA content of cells of *Escherichia coli* strain B and its radio-resistant mutant, strain B/r, in the logarithmic phase of growth are not statistically significant. In the stationary phase, cells of strain B/r contain more DNA than those of strain B, but the difference is barely significant; the RNA content of the B/r cells is significantly higher. The differing radiosensitivities of the two strains cannot be correlated with differences in their content of nucleic acids.

INTRODUCTION

In recent years considerable emphasis has been placed on the possible connection between DNA and the specific site of damage in living cells exposed to ionizing or u.v. radiations. It is sometimes assumed that the lethal effect of radiation (*i.e.* inhibition of clone formation) is primarily due to damage to the DNA itself. Direct correlation between the DNA content of cells and their radiosensitivity might support this assumption, although lack of correlation is, of course, not evidence against it. Several authors have attempted to relate radiosensitivity to DNA content. Thus PARDEE AND PRESTIDGE[1] concluded that a relative surplus of DNA over protein and RNA in cells of *E. coli* B was probably responsible for reducing the u.v. sensitivity of bacteria which had been treated with β-2-thienylalanine. BILLEN[2] implied that the reduction of X-ray sensitivity, conferred by incubating *E. coli* B/r and *E. coli* 15T$^-$ with chloramphenicol before irradiation, was due to an increase in the DNA/protein ratio of the cells. These papers exemplify the line of reasoning that the radio-

Abbreviations: DNA, deoxyribonucleic acid; RNA, ribonucleic acid.