

larly protein-restricted during pregnancy suffer from retardation of kidney development and altered kidney function. They may also lack the vigor to suckle (9). Lee and Chow (10) have reported that the restricted progeny showed reduced feed efficiency and low nitrogen balance; they excreted more amino acids than the controls. Thus, such progeny (F_1) may indeed suffer from *cryptic malnutrition*, even when postnatally given full access to normal food (groups B and C). Groups A, D, and E may represent a more overt malnutrition in F_1 , since their nursing mothers were on a protein-restricted diet. In both cases, the progeny (F_2) of F_1 had a cerebral cell deficiency (Table 1), in accordance with our original findings (1).

Many investigators have implied that protein deprivation before and after birth results in mental impairment and brain cell deficiency in children [for reviews see (2, 11)]. If the results with rats have a bearing on the situation in humans, one must consider that, even after nutritional rehabilitation, a cerebral deficiency may last for at least one generation longer.

STEPHEN ZAMENHOF
EDITH VAN MARTHENS
LUDMILA GRAUEL

Department of Medical Microbiology
and Immunology, Department of
Biological Chemistry, Brain Research
Institute, and Mental Retardation
Center, University of California
School of Medicine, Los Angeles 90024

References and Notes

1. S. Zamenhof, E. van Marthens, F. L. Margolis, *Science* **160**, 322 (1968).
2. M. Winick, *J. Pediatr.* **74**, 667 (1969).
3. F. J. Zeman and E. C. Stanbrough, *J. Nutr.* **99**, 274 (1969).
4. J. J. Cowley and R. D. Griesel, *Anim. Behav.* **14**, 506 (1966).
5. F. Wehmer, R. H. Porter, B. Scales, *Nature* **227**, 622 (1970).
6. S. Zamenhof, H. Bursztyn, K. Rich, P. J. Zamenhof, *J. Neurochem.* **11**, 505 (1964); F. L. Margolis, *ibid.* **16**, 447 (1969); S. Zamenhof, E. van Marthens, H. Bursztyn, in *Hormones in Development*, M. Hamburg and E. J. W. Barrington, Eds. (Appleton-Century-Crofts, New York, in press); S. Zamenhof and E. van Marthens, in *Cellular Aspects of Neural Growth and Differentiation*, D. C. Pease, Ed. (Univ. of California Press, Berkeley, 1971), pp. 329-359.
7. F. J. Zeman, *J. Nutr.* **94**, 111 (1968).
8. S. M. Hall and F. J. Zeman, *ibid.* **95**, 49 (1968).
9. F. J. Zeman, *ibid.* **93**, 167 (1967).
10. C. J. Lee and B. F. Chow, *ibid.* **94**, 20 (1968).
11. R. Barnes, *Fed. Proc.* **26**, 144 (1967); D. Baird, *ibid.*, p. 134.
12. Supported by NIH grants HD-04612 and NS-08723, U.S. Office of Education grant OEG 9-70-0043(057)00705, and Nutrition Foundation grant 409.

28 December 1970

21 MAY 1971

DNA Polymerase Required for Rapid Repair of X-ray-Induced DNA Strand Breaks in vivo

Abstract. A much higher yield of DNA single-strand breaks was obtained in the DNA polymerase-deficient mutant *Escherichia coli* K-12 *pol A1* after a given dose of x-rays than had been found before in *Escherichia coli*. The increased yield of single-strand breaks was due to the absence of a rapid repair system, which had not been described in *Escherichia coli* K-12. This absence probably accounts for the x-ray sensitivity of the *pol A1* mutant. The rapid repair system can be reversibly inhibited in *pol+* cells.

There is currently a dichotomy in the published data concerning the radiation energy required to produce one single-strand break in DNA. Although many authors favor ~60 ev per break [see summary in (1)], those who have worked with the chromosome of *Escherichia coli* have all (2-7) obtained data quantitatively similar to those of McGrath and Williams (2), which yield values of 300 to 600 ev per break. Alexander *et al.* (8) have shown that in *Micrococcus radiodurans* approximately 90 percent of the DNA single-chain breaks produced by aerobic x-irradiation are rapidly repaired in buffer (pH 8.6) at 30°C but not at 0°C; the remaining breaks are repaired when the cells are subsequently incubated in growth medium at 30°C.

These observations with *M. radiodurans* have led to the suggestion (1) that in *E. coli* many of the DNA single-strand breaks are rapidly repaired before the samples can be analyzed by sedimentation. However, a greater number of breaks were not detected even when *E. coli* were held at 0°C under normal experimental conditions (phosphate buffer, pH 7) from the start of irradiation until their lysis on the alkaline sucrose gradient (2, 5), an observation that we have confirmed in this laboratory.

Radiation studies on the DNA polymerase-deficient mutant, *pol A1* (9), indicated that it was very sensitive to killing by aerobic x-irradiation, and there was an unexpectedly high yield of DNA single-strand breaks (for a given dose of x-rays) compared with the parent strain (10). This suggested that *pol A1* might be defective in a "rapid repair" system which had not been demonstrated in *E. coli*. In confirmation of this we observed that in *E. coli pol+* cells the apparent energy requirement for the production of a single-strand break in DNA is dependent upon the irradiation conditions and the time taken thereafter to lyse the

cells. When irradiated aerobically at 0°C in 0.05M phosphate buffer at pH 8.0, and lysed within about 1 minute of the end of irradiation, *pol+* cells showed a requirement of 80 to 140 ev per break—about five times lower than previously observed at room temperature and pH 6.9 (3, 7)—and this energy requirement was comparable with that observed for *pol A1* cells. The yield of breaks in *pol A1* cells was not affected by these different irradiation conditions, nor by the speed with which subsequent lysis was carried out. These results suggest that the *pol A* gene controls a repair system which rapidly rejoins x-ray-induced DNA single-chain breaks, even in buffer.

The DNA polymerase-deficient mutant *E. coli* K-12 P3478 (*pol A1*) (9)

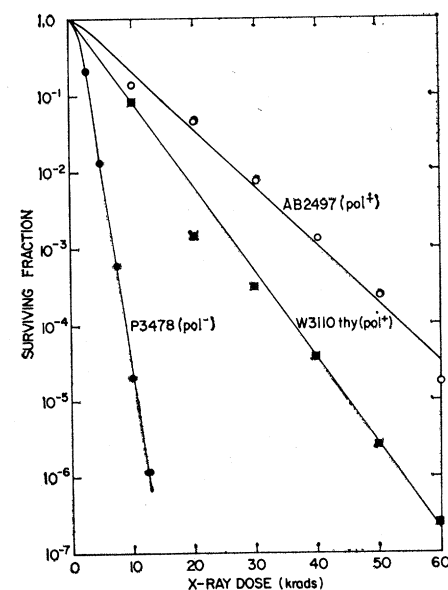


Fig. 1. Survival curves of *E. coli* K-12 *pol+* (W3110 *thy* and AB2497) and *pol-* (P3478) cells exposed to 50-kv (peak) x-rays. Cells in exponential growth in glucose-salts-BCA medium were collected at $\sim 10^8$ cell/ml, washed, resuspended, and irradiated at room temperature ($\sim 25^\circ\text{C}$) in aerated 0.05M phosphate buffer, pH 6.9. They were then diluted in water and plated on glucose-salts-BCA-agar plates.

and its progenitor W3110 *thy* were used in most of our experiments. In some experiments AB2497 (11) was also used as a *pol*⁺ control.

Cells were grown to log phase (0.5 to 1.0×10^8 cell/ml) in a glucose-salts medium (12) supplemented with Bacto Casamino Acids (BCA; Difco) (1 mg/ml) and thymine ($5 \mu\text{g/ml}$); [^3H]thymine ($25 \mu\text{C/ml}$) (New England Nuclear, 13.6 c/mmole) was also added. For AB2497 the medium also contained the required amino acids (threonine, leucine, arginine, histidine) at 1.0 mmole/liter and vitamin B₁ (thiamine hydrochloride) at $0.5 \mu\text{g/ml}$. Survival determinations were made by plating cells on the same medium solidified with 0.9 percent Oxoid agar-agar No. 3. The cells were harvested by Millipore filtration ($0.45 \mu\text{m}$), washed, and re-suspended in the appropriate buffer. Irradiations were carried out aerobically, with a twin tube 50-kv (peak) x-ray unit (13) with 0.2 or 0.3 mm of alumi-

num for added filtration, at 8 to 9 krad/min, depending upon the volume of the sample. In our experiments, the cells were lysed immediately after irradiation by layering them directly onto a cap of 0.5N NaOH and 0.5 percent Sarkosyl (Geigy NL30) (4) on top of an alkaline sucrose gradient (5 to 20 percent; weight/volume). Apart from the omission of the lysozyme treatment, the sedimentation procedure and analysis of results have been described (3). In the experiments with AB2497 the centrifugation time was reduced from 120 to 105 minutes.

Typical survival curves for the three strains of *E. coli* K-12 are shown in Fig. 1. The D_0 values (dose to reduce survival by a factor of e on the exponential part of the survival curve) are 0.82, 3.83, and 5.70 krad for the three strains P3478, W3110 *thy*, and AB2497, respectively.

Figure 2 shows the sedimentation characteristics of DNA from these strains when cells were lysed immediately after x-irradiation under two different conditions; one was 0.05M phosphate buffer, pH 6.9, at room temperature ($\sim 25^\circ\text{C}$), and the other was 0.05M phosphate buffer, pH 8.0, at 0°C (14). The sedimentation profile of the DNA from unirradiated cells remained the same, regardless of the pH or temperature of the buffer in which the cells were suspended prior to lysis. The DNA from rapidly lysed *pol*⁺ cells irradiated at 0°C sediments more slowly (contains more single-strand breaks) than DNA isolated from cells irradiated at room temperature. The DNA from *pol*⁻ cells shows the same sedimentation characteristics (contains the same number of breaks) under both conditions. We can calculate (3) the apparent number of breaks resulting from a particular dose of radiation, and hence the apparent energy required to produce each DNA single-strand break under certain experimental conditions. The data shown in Fig. 2 indicate an apparent energy requirement in W3110 *thy* of 80 ev per break after irradiation at 0°C , pH 8.0, and 266 ev per break after irradiation at room temperature, pH 6.9. In P3478 (Fig. 2b) the apparent energy requirement is 59 ev per break under both sets of conditions. The data for AB2497 (Fig. 2c) indicate a requirement of 141 ev per break at 0°C , pH 8.0, and 413 ev per break at room temperature, pH 6.9. At 0°C and pH 8.0, both *pol*⁺ strains showed more DNA single-strand

breaks in the presence of 0.1M ethylenediaminetetraacetate (EDTA) than in its absence, while the yield of breaks in *pol*⁻ cells was unchanged. In the presence of EDTA, both *pol*⁺ and *pol*⁻ cells showed approximately the same energy requirement to produce one DNA single-strand break (15).

In order to demonstrate that the low number of breaks seen in *pol*⁺ cells at room temperature probably arises from a high initial yield followed by a rapid repair, the following procedure was adopted. A suspension of *pol*⁺ cells was irradiated at 0°C and a portion was lysed immediately. The remainder of

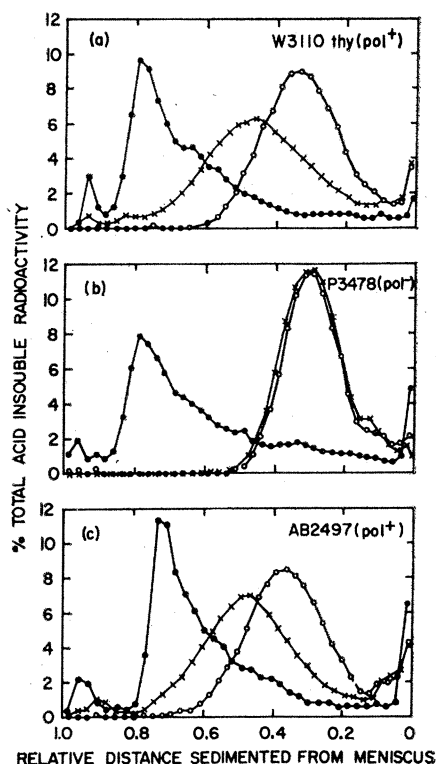


Fig. 2. Sedimentation of DNA from *E. coli* K-12 *pol*⁺ (W3110 *thy*), *pol*⁻ (P3478), and *pol*⁺ (AB2497) after aerobic irradiation with 16.3 krad followed by immediate lysis. (a), *pol*⁺ (W3110 *thy*); (b), *pol*⁻ (P3478); and (c), *pol*⁺ (AB2497). The cells were irradiated in 0.05M phosphate buffer at pH 6.9 and 25°C or at pH 8.0 and 0°C . The centrifugation time in (c) was reduced from 120 minutes to 105 minutes. ●, Unirradiated control; x, irradiated at pH 6.9 at 25°C ; ○, irradiated at pH 8.0, 0°C .

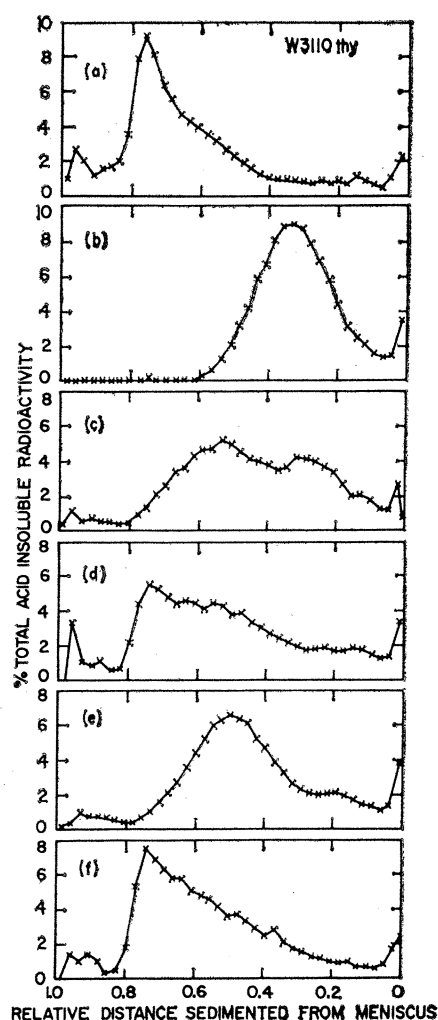


Fig. 3. Repair of DNA single-strand breaks in *E. coli* K-12 *pol*⁺ (W3110 *thy*) cells x-irradiated aerobically under different conditions. Cells suspended in 0.05M phosphate buffer, pH 8.0: (a), unirradiated control; (b), 16.3 krad at 0°C , immediate lysis; (c), 16.3 krad at 0°C , 15 minutes at 25°C before lysis; (d), 16.3 krad at 0°C , 45 minutes in growth medium at 37°C before lysis; (e), 16.3 krad at 25°C , immediate lysis; (f), 16.3 krad at 25°C , 45 minutes in growth medium at 37°C before lysis.

the suspension was allowed to stand for 15 minutes at room temperature, after which another portion was lysed on another gradient. The remainder of the suspension was then added to growth medium and incubated at 37°C for 45 minutes before lysis. These results were compared with those from cells irradiated under similar conditions at room temperature and either lysed immediately, or lysed after 45 minutes of incubation at 37°C in growth medium. The results for W3110 *thy* and AB2497 (Figs. 3 and 4, respectively) show that there are two general phases in the repair of x-ray-induced DNA strand breaks. The first phase takes place rapidly in buffer at room temperature (compare Fig. 3b with 3c and Fig. 4b with 4c), and produces a DNA sedimentation profile that is similar to that obtained when the sample has been irradiated at room temperature (Figs. 3e and 4e). Upon further incubation at 37°C in growth medium the profiles are restored to control positions (Fig. 3, d and f; Fig. 4, d and f).

The profiles in Fig. 4, c and e, are not identical, presumably due to the fact that sample 4e was lysed about 1 minute after the end of the irradiation (duration 2 minutes) while 4c was allowed to stand for 15 minutes at room temperature. The rapid (buffer) repair is probably not complete in the shorter period of time, so that more repair has taken place in sample 4c than in 4e.

Although the reincubated samples of AB2497 (Fig. 4, d and f) show a profile similar to that for the unirradiated control (Fig. 4a), this is not true for W3110 *thy*. The failure of complete repair to take place either in the buffer stage (Fig. 3c) or the growth medium stage (Fig. 3d) in W3110 *thy* may be associated with the tendency for cells of this strain to die if held in buffer after irradiation. In repeated survival curves, several points have consistently fallen below the anticipated line. These were typically the intermediate dose points, and were for those cells which were plated last. In Fig. 1 the 20- and 30-krad points demonstrate this effect. Keeping irradiated W3110 *thy* cells in buffer for too long thus appears to impair their ability both to survive and to repair their DNA (note unrepaired peak of DNA in Fig. 3c). Cell death can be partly prevented by the presence of 0.3M sucrose in the buffer.

Despite the lack of complete repair in W3110 *thy*, both *pol*⁺ strains showed a different number of strand breaks

when assayed at 25°C, pH 6.9 or 8.0, and at 0°C, pH 8.0; and in both strains the number of observable breaks (at 0°C) was reduced when the cells were held in buffer at room temperature after irradiation. There were no observable differences in the DNA from *pol*⁻ cells after irradiation under either condition (Fig. 2b), suggesting that the buffer repair system is absent in this *pol*⁻ strain.

Single-chain breaks in DNA appear to be lethal lesions in those strains of *E. coli* that are defective in the repair of chain breaks (2, 7). Mutants deficient in genetic recombination (*rec*) are very

sensitive to x-irradiation (D_0 for *rec A* is 1.1 krad), and are deficient in the repair of x-ray-induced single-chain breaks (7). Thus, the *rec* genes not only control genetic recombination but also a mechanism for the slow repair (~40 minutes at 37°C) of x-ray-induced chain breaks. The DNA polymerase-deficient mutant (*pol A1*) has an x-ray sensitivity ($D_0 = 0.82$ krad) which is comparable to that of the recombination deficient mutant, *rec A*. From the sucrose gradient data presented here we suggest that the *pol A* gene controls a mechanism for the rapid repair of x-ray-induced single-chain breaks (16). Thus *E. coli* appears to possess repair systems for aerobic x-ray damage which may be similar to the two systems (fast and slow) described for *M. radiodurans* (8).

The presence of *pol* repair in most strains of *E. coli*, and the relative difficulty with which it is inhibited, enables us to explain the discrepancy between the radiation energy apparently required to produce single-strand breaks in the chromosome of *E. coli*, compared to most other systems studied. In *pol A1* cells and in inhibited *pol*⁺ cells, the value of ~75 ev per break (15, 17) agrees well with the majority of the data published for other systems (1).

CHRISTOPHER D. TOWN

KENDRIC C. SMITH

HENRY S. KAPLAN

Department of Radiology, Stanford
University School of Medicine,
Stanford, California 94305

References and Notes

1. C. J. Dean, M. G. Ormerod, R. W. Serianni, P. Alexander, *Nature* **222**, 1042 (1969).
2. R. A. McGrath and R. W. Williams, *ibid.* **212**, 534 (1966).
3. C. D. Town, K. C. Smith, H. S. Kaplan, *J. Bacteriol.* **105**, 127 (1971).
4. D. M. Ginsberg and H. K. Webster, *Radiat. Res.* **39**, 421 (1969).
5. P. M. Achey and V. G. Whitfield, *J. Bacteriol.* **95**, 1180 (1968).
6. H. S. Kaplan, *Proc. Nat. Acad. Sci. U.S.A.* **55**, 1442 (1966).
7. D. S. Kapp and K. C. Smith, *J. Bacteriol.* **103**, 49 (1970).
8. P. Alexander, C. J. Dean, A. R. Lehmann, M. G. Ormerod, P. Feldschreiber, R. W. Serianni, in *Radiation Protection and Sensitization*, H. L. Moroson and M. Quintiliani, Eds. (Barnes & Noble, New York, 1970), pp. 15-34.
9. P. de Lucia and J. Cairns, *Nature* **224**, 1164 (1969). We thank Dr. John Cairns for making this strain available to us.
10. This initial observation was made by Dr. A. K. Ganesan, whom we also thank for advice and discussions. While this communication was in preparation, T. Kato reported similar observations on *E. coli res* (R15), an x-ray sensitive, DNA polymerase-deficient derivative of *E. coli B* [*Gamma Field Symposia*, (National Institute of Radiation Breeding, Ohmiya, Ibaraki, Japan, 5 1970), No. 8, p. 1]. Some of these data also appear in T. Kato and S. Kondo [*J. Bacteriol.* **104**, 871 (1970)]. They observed the same yield of x-ray-induced DNA single-chain breaks in both *res*⁺ and *res*⁻ cells after x-irradiation at

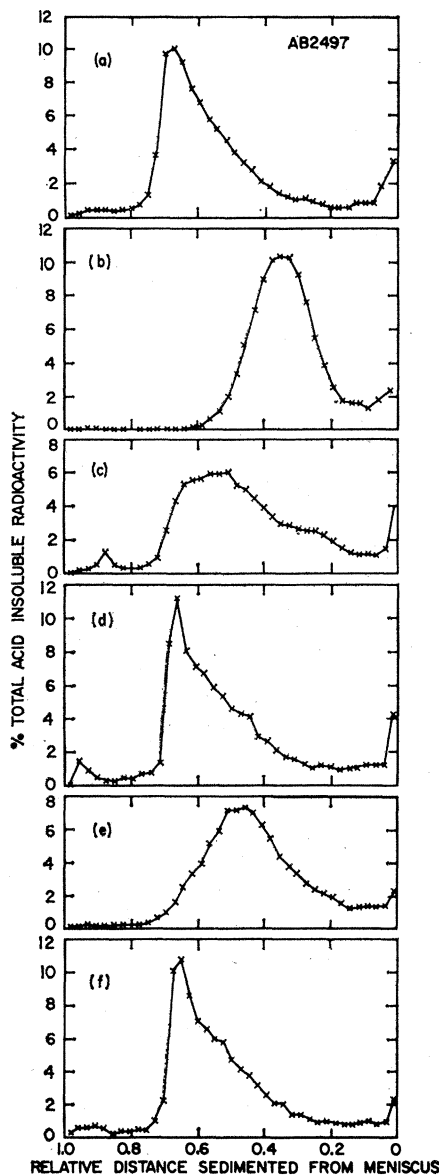


Fig. 4. Repair of DNA single-strand breaks in *E. coli* K-12 *pol*⁺ (AB2497) cells x-irradiated under different conditions. The experimental design is the same as described in Fig. 3 except that the centrifugation time was reduced from 120 to 105 minutes.

-21°C, but a threefold higher yield in the *res-* cells (versus *res+*) when not frozen. On reincubation of irradiated *res-* cells in growth medium, the DNA chain breaks were not repaired and the cells showed extensive DNA degradation. The molecular weight of denatured (but not native) DNA decreased significantly. We have not observed this type of degradation in *E. coli* K-12 *pol A1*. Kato and Kondo offer two alternative hypotheses to account for the x-ray sensitivity of their mutant: (i) "excessive endonucleases" and (ii) "polymerase participates in the repair of X-ray damage." Our own data demonstrate that DNA polymerase is necessary for the fast repair of DNA single-strand breaks.

11. P. Howard-Flanders, R. P. Boyce, L. Theriot, *Genetics* 53, 1119 (1966).
12. A. K. Ganesan and K. C. Smith, *J. Bacteriol.* 96, 365 (1968).
13. R. Loevinger and P. Huisman, *Radiat. Res.* 24, 357 (1965).
14. Phosphate buffer, pH ~7, has been used in studies on *E. coli* which demonstrate a low yield of x-ray-induced DNA single-strand breaks. Barbitol buffer, pH 8.6, was used in the work on *M. radiodurans* in which a higher number of chain breaks was found. These data suggested that a shift to pH 8

might be an important factor in controlling the rate of *pol* repair, particularly at 0°C. This proved to be so. We also observed that after irradiation in tris(hydroxymethyl)amino-methane (tris) and barbitol buffers, inhibition was less reversible (by raising the temperature) than in phosphate buffer.

15. The mean energy requirement to produce one DNA single-strand break in *pol-* and inhibited *pol+* (W3110 *thy*) cells is 75 ev per break, and was determined from 22 measurements in 11 experiments.
16. The *pol-rec-* double mutant (which would presumably be devoid of any repair capacity of the kind discussed here) is thought to be inviable (J. D. Gross, *Int. Congr. Microbiol. Rep. Proc.* 10th, Mexico City, August 1970).
17. S. Lehnert and H. Moroson have reported [*Radiat. Res.* 45, 299 (1971)] a value of 87 ev per break for log phase *E. coli* B/r CSH irradiated in cold 0.01M tris buffer (pH 8.0) in the presence of 1 mM EDTA.
18. Supported by PHS grants CA-6437 and CA-10372 and a research career development award CA-3709 (to K.C.S.) from the National Cancer Institute. We thank Priscilla Mather for technical assistance.

23 November 1970; revised 18 January 1971 ■

Cross-Linked Transfer RNA Functions in All Steps of the Translation Process

Abstract. A specific photochemical reaction between 4-thiouridine and cytosine cross-links two arms of transfer RNA. This cross-link, introduced into phenylalanine transfer RNA and arginine transfer RNA, limits the conformational freedom of the molecule. Both modified transfer RNA's are capable of functioning in all steps of protein synthesis with this restraint on allowable conformations.

The role of tRNA (1) as an adaptor molecule for translation of a genetic message into a protein sequence requires highly specific interactions between tRNA and various enzymes, messenger RNA, and ribosomes. The steps necessary for the total translation

process in which tRNA is involved include enzymatic aminoacylation of tRNA, binding of GTP and aminoacyl tRNA to T factor, binding to the ribosomal site for aminoacyl-tRNA, peptide bond formation, translocation of mRNA-polypeptidyl-tRNA complex to

the peptidyl-tRNA binding site on the ribosome, and release of tRNA from the ribosome. This multitude of functional steps involving tRNA has led to proposals that changes in the macromolecular conformation of tRNA may be required for some of these steps. Through studies of the longitudinal relaxation of water protons enhanced by paramagnetic manganous ions bound to tRNA, Cohn *et al.* (2) suggested that tRNA conformation changes on aminoacylation. In proposing the reciprocating ratchet model for translation, Woese (3) suggests that tRNA may pass through several conformational states.

We have investigated the question of whether a chemical cross-link between the positions 8 and 13 in phenylalanine tRNA and arginine tRNA modifies the ability to function in a total protein synthesis system. Favre *et al.* (4) have reported for tRNA^{Val}, tRNA^{Val}, tRNA^{Met}, tRNA^{fMet}, and tRNA^{Phe} a specific modification of 4-thiouridine in the position 8. The reaction requires a cytosine in position 13. This modification is unique in that it makes a cross-link between the amino acid acceptor stem and the dihydrouridine-containing loop of the cloverleaf model of tRNA. Chemical cross-linkage of nonadjacent nucleotides in tRNA can reduce the number of allowed conformational states in a tRNA molecule.

We have shown that this photochemical reaction occurs readily in *Escherichia coli* tRNA^{Arg} (5). The prog-

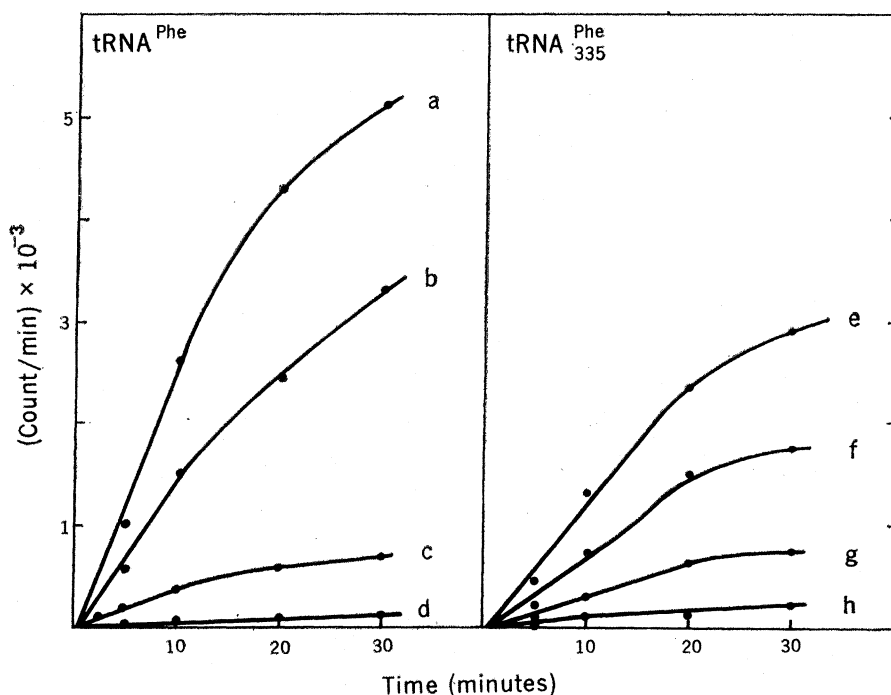


Fig. 1. [¹⁴C]Phenylalanine incorporation in response to added tRNA^{Phe} and tRNA^{Phe 335}. A 1-ml reaction mixture contained 50 μmole of tris buffer (pH 7.2), 15 μmole of MgCl₂, 30 μmole of NH₄Cl, 3 μmole of ATP, 0.2 μmole of GTP, 5 μmole of phosphoenolpyruvate, 20 μg of pyruvate kinase, 10 μmole of mercaptoethanol, 10 to 12 A₂₆₀ units (absorbance at 260 nm) of ribosomes, and as much as 3 mg of S 100 protein (10). In addition, for phenylalanine incorporation the reaction mixture contained 100 μg of poly(U) and 17.4 nmole [¹⁴C]phenylalanine (specific activity 28.6 μC/μmole). The incubations were at 37°C with variable amounts of tRNA^{Phe} and tRNA^{Phe 335} added. Incorporation of phenylalanine into polypeptide linkage was measured as product insoluble in 5 percent trichloroacetic acid after release from tRNA by incubation in alkali. Curves a, b, c, d represent 2.33, 1.16, 0.09, and 0.0 A₂₆₀ units of tRNA^{Phe} per milliliter added, respectively, to the incubation mixture. Curves e, f, g, and h represent 2.43, 1.22, 0.28, and 0.0 A₂₆₀ units of tRNA^{Phe 335} per milliliter added, respectively, to the reaction mixture.