high degree of rigidity. The stoichiometry of interaction between hapten and antibody was determined by an electron spin resonance titration, in agreement with the results obtained from a fluorescence titration. It appears likely that the spin-labeling method will be useful in studies of the interaction of haptens, coenzymes, inhibitors, and substrates with proteins and other macromolecules.

We are indebted to Professor Harden M. McConnell for numerous stimulating discussions and encouragement in this work. We wish to thank Mrs. Diane Griffin for her gift of antibody. It is a pleasure to acknowledge the capable technical assistance of Miss Verena Kurer.

* This work was supported by grants from the National Science Foundation (GB 2081 and GP 3430) and the National Institutes of Health (GM 11783).

† NAS-NRC postdoctoral fellow (supported by the U.S. Air Force Office of Scientific Research). Present address: Department of Chemistry, University of Oregon, Eugene

[1] Ohnishi, S., and H. M. McConnell, *J. Am. Chem. Soc.*, **87**, 2293 (1965).

[2] Stone, T. J., T. Buckman, P. L. Nordio, and H. M. McConnell, these PROCEEDINGS, **54**, 1010 (1965).

[3] Rozantzev, E. G., and M. B. Neiman, *Tetrahedron*, **20**, 131 (1964).

[4] Rozantzev, E. G., and L. A. Krinitzkaya, *Tetrahedron*, **21**, 491 (1965).

[5] Weber, G., *Biochem. J.*, **51**, 155 (1952).

[6] Velick, S. F., C. W. Parker, and H. N. Eisen, these PROCEEDINGS, **46**, 1470 (1960).

[7] Stryer, L., *J. Mol. Biol.*, **13**, 482 (1965).

[8] Hundley, L., and L. Stryer, to be published.

[9] Hoffmann, A. K., and A. T. Henderson, *J. Am. Chem. Soc.*, **83**, 4671 (1961).

[10] McConnell, H. M., C. Heller, T. Cole, and R. W. Fessenden, *J. Am. Chem. Soc.*, **82**, 766 (1960).

[11] Griffith, O. H., D. Cornell, and H. M. McConnell, *J. Chem. Phys.*, **43**, 2909 (1965).

[12] Kivelson, D., *J. Chem. Phys.*, **33**, 1094 (1960).

[13] Freed, J. H., and G. K. Fraenkel, *J. Chem. Phys.*, **39**, 326 (1963).

[14] Jabloński, A., *Bull. Acad. Polon. Sci., Ser. Sci. Math. Astron. Phys.*, **8**, 259 (1960).

[15] It is of interest to compare rotational relaxation times determined independently by ESR and fluorescence polarization methods. A study of spin-labeled fluorescent molecules will be published in detail elsewhere by O. H. Griffith, D. Cornell, and L. Stryer.

[16] Krause, S., and C. T. O'Konski, *Biopolymers*, **1**, 503 (1963).

---

*IN VITRO STUDIES ON THE MECHANISM OF SUPPRESSION OF A NONSENSE MUTATION**

BY DEAN L. ENGELHARDT, ROBERT E. WEBSTER, ROBERT C. WILHELM, AND NORTON D. ZINDER

THE ROCKEFELLER UNIVERSITY AND YALE UNIVERSITY

Communicated by E. L. Tatum, November 5, 1965

On the basis of genetic and physiological analyses, Benzer and Champe[1] and Garen and Siddiqi[2] concluded that suppression of nonsense mutations occurred at the level of translation of messenger RNA into protein. It remained to be shown which, if any, of the known components of protein synthesis were responsible for suppression. The present report describes the use of an *in vitro* assay for suppression to identify a suppressor component. The development of this assay depended on the following observations. (1) Unsuppressed nonsense mutations cause the premature termina-

tion of growing protein chains.[3] (2) RNA from phage f2 directs the synthesis, in extracts of *Escherichia coli*, of a polypeptide product corresponding to the coat protein of the phage particle.[4] A mutant form of this phage has been isolated which contains a nonsense mutation in the gene specifying the coat protein.[5] (3) Suppression of a nonsense mutation by the bacterial suppressor gene, Su-1, results in the insertion of serine into the position in a protein molecule which is affected by the mutation. This result has been obtained for proteins as diverse as alkaline phosphatase,[6] T4 head protein,[7] and f2 coat protein.[5]

The above observations suggested that f2 RNA might be used in an *in vitro* assay for suppression. This communication will demonstrate that sRNA is the component of the protein-synthesizing machinery responsible for genetic suppression. This sRNA leads to the insertion of the amino acid serine into the coat protein of the phage, thereby preventing premature chain termination. The same conclusion has been reached by Capecchi and Gussin,[8] using a similar system.

Materials and Methods.—Bacteria and phage: S26, a nonsuppressing (Su^-) strain of *E. coli* K12; S26Rle, a suppressing (Su^+) strain carrying the Su-1 gene;[6] f2, the wild-type RNA phage; Sus4A, a mutant of f2 which has a nonsense mutation in the gene that specifies the coat protein.[5]

In vitro incorporation: The S-30 system described by Schwartz (1965)[9] was employed, in which 0.03 *M* NH_4^+ was used in place of K^+. The reaction mixture was preincubated for 10 min before the addition of radioactive amino acid and phage RNA. Incubations were carried out at 34°. The amount of phage RNA added was in the range of 150–300 μg for each ml of reaction mixture. Approximately 10–15 lysine-C^{14}-molecules were incorporated into TCA-insoluble material per molecule of phage RNA added.

Enzymatic digestion and peptide analysis: Enzymatic digestion of protein with chymotrypsin was performed as described by Notani *et al.*[5] The *in vitro* protein product was first desalted either by repeated 5% TCA washings or by passage through a Sephadex G-25 column (30 × 1.8-cm) in 50% acetic acid. The chymotryptic peptides were separated either by fingerprinting on paper or by column chromatography with Dowex-1 × 2. The first dimension of the fingerprints was electrophoresis in pH 1.9 formic-acetic acid buffer for 2.5 hr under varsol.[5] For the second dimension, ascending chromatography with a butanol-acetate solvent (5 *n*-butanol:2 acetic acid:2 water equilibrated at 37° for 72 hr) was employed. Radioautography of the fingerprint was performed as described elsewhere.[5]

The amount of isotope label present in peptides was determined as follows. The relevant areas of the fingerprint were cut into 1.5-cm squares. The label was eluted by incubating the paper squares in 5 ml 88% formic acid for 30 min in glass liquid-scintillation vials. The papers were removed, and the formic acid was evaporated under vacuum at 70°. The dried residue was resuspended in 0.15 ml of formic acid, 15 ml of Bray's solution (100 gm naphthalene, 7 gm PPO, and 0.05 gm POPOP per liter of diethylene oxide) was added, and the sample was counted in a Nuclear-Chicago model 6804 scintillation counter. The efficiency of extraction was about 95%.

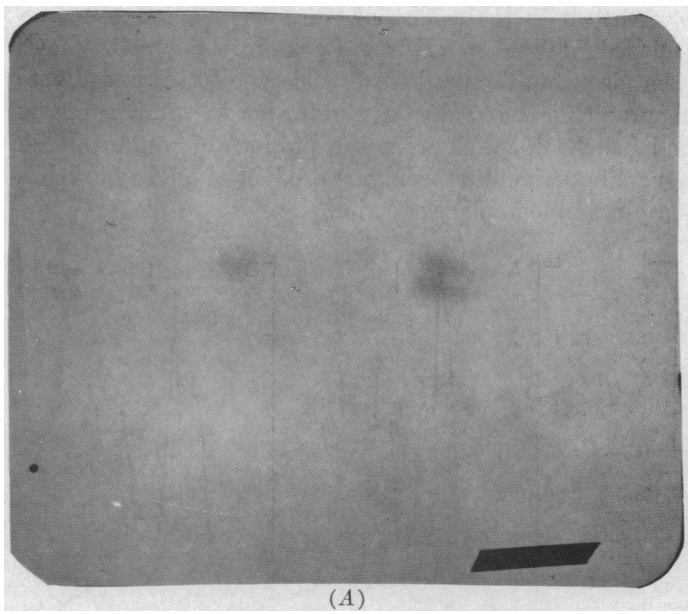
The chymotryptic peptides were also separated by attaching them to a 0.9 × 18-cm Dowex-1 column (Bio-Rad AG 1 × 2,200–400 mesh) at 60° and eluting with 30 ml of 3% pyridine, followed by a gradient of 250 ml 3% pyridine to 250 ml of 2 *M* pyridine acetate (161 ml pyridine and 143 ml glacial acetic acid/liter). An unlabeled tripeptide was used as a reference maker on the column. Samples of 1.5 ml were collected, and aliquots of each sample were evaporated to dryness. The residues were incubated at 60° for 20 min with 0.5 ml of 25% hydroxide of hyamine in methanol. Twenty ml of scintillation fluid (7 gm PPO and 0.05 gm POPOP per liter of toluene) was added and the samples were counted in a scintillation counter.

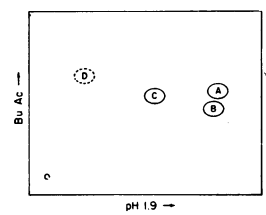
Preparation of sRNA: The sRNA was prepared and assayed for acceptor activity according to the procedure of Zubay,[10] except that saponification was carried out in water at pH 8.6, at 37° for 2 hr. The serine acceptor activity was in the range of 1–2 mμmoles of serine per mg of sRNA isolated from both the Su^- and Su^+ bacterial strains.

Isolation of coat protein fragment: The sample was prepared for gel-filtration by extracting the reaction mixture for 30 min with an equal volume of glacial acetic acid. The precipitate which

formed was removed by centrifugation and then re-extracted with 1/4 vol of 50% acetic acid and again centrifuged. The supernatants were pooled, evaporated *in vacuo* to 1 ml, and placed on a column which contained a top layer of Biogel P4 (30 × 1.8 cm) and then a layer of Sephadex G-25 fine (70 × 1.8 cm) in 50% acetic acid. Flow rate was 5–6 ml per hr; 1.5-ml samples were collected, and aliquots counted in a scintillation counter.

Results.—Nonsense mutations of the class sus-3 are located in the gene that specified the coat protein. Suppression of these mutations results in a substitution of serine for a glutamine residue in the N-terminal tryptic peptide (33 residues). Chymotryptic digestion of this peptide releases a peptide of sequence thr-gluNH_2-phe from the wild-type protein and thr-ser-phe from the mutant protein. The two peptides can be resolved by electrophoresis at pH 1.9; the serine-containing peptide moves about 5 per cent faster than the glutamine-containing peptide.[5] The phenylalanine in these peptides can be used as a diagnostic marker. Figure 1*A* is a





(*B*)

FIG. 1.—Radioautogram and schematic illustration of phenylalanine containing chymotryptic peptides of f2 coat protein. (*A*) A fingerprint of digest coat from purified phage. (*B*) A diagram of the fingerprint from a mixture of C^{14}-labeled purified phage (*A*, *B*, and *C*), and H^3-labeled product from an Su^+ extract (*A* and *D*).

radioautogram of a two-dimensional fingerprint of a chymotryptic digest of C^{14}-phenylalanine-labeled f2 protein that was isolated from purified phage. The radioautogram is shown schematically in Figure 1*B*; there are three phenylalanine peptides, labeled *A*, *B*, and *C*. Peptide *C* is thought to be a nonapeptide, consisting of part of the tryptic peptide N-2.[5] Peptide *B* is a tetrapeptide of sequence ala-ser-aspNH_2-phe which is the N-terminal peptide of the coat protein (Konigsberg and Zinder, unpublished data). Peptide *A* is the tripeptide thr-gluNH_2-phe. The mutant tripeptide would be located at the same chromatographic position as peptide *A* but to the right of peptide *B*.[5]

Fingerprints of the *in vitro* product stimulated by wild-type f2 RNA reveal a peptide which is congruent with peptide *A*. This is shown in Figure 1*B*, which represents the fingerprint of a mixture of C^{14}-phenylalanine-labeled *in vivo* protein and H^3-phenylalanine protein labeled *in vitro*. Spots *A*, *B*, and *C* contain C^{14} from the *in vivo* material, and spots *A* and *D* contain H^3 from the *in vitro* material. Spot

A is the tripeptide and is common to both materials. Spot *D* is known to be a derivative of the tetrapeptide, spot *B*, which has an altered electrophoretic mobility. Further results supporting this last point will appear in a subsequent publication.

The difference in the tripeptide specified by wild-type and mutant phage RNA can be demonstrated in fingerprints of the *in vitro* products. Chymotryptic digests of the product obtained with mutant RNA in Su^+ extracts yielded a tripeptide distinct from that produced with wild-type RNA. Figure 2 diagrams this difference. The product from wild-type RNA was labeled with C^{14}-phenylalanine, and the product from mutant RNA was labeled with H^3-phenylalanine. The two products were mixed, digested, and fingerprinted. Spot *A* contained the C^{14} (wild-type) tripeptide and spot *A'* the H^3 (mutant) tripeptide. The positions of *A* and *A'* correspond to those expected for the glutamine and serine containing tripeptides, respectively. Direct evidence that peptide *A'* contains serine has been obtained by labeling the *in vitro* product with both H^3-serine and C^{14}-phenylalanine and chromatographing the doubly labeled material on a Dowex-1 column. Figure 3*A* shows the elution profile of the peptides from the product of wild-type RNA, and Figure 3*B* shows the corresponding profile for the product of mutant RNA. Serine appears only in the phenylalanine-labeled tripeptide from the product of mutant RNA and is absent in the corresponding tripeptide from the product of wild-type RNA (which presumably contains glutamine in place of serine).

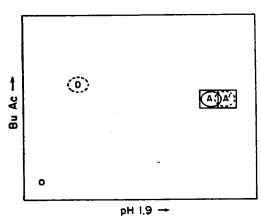


FIG. 2.—Schematic illustration of major soluble phenylalanine containing chymotryptic peptides of *in vitro* product. Wild-type RNA-stimulated product C^{14}-labeled is mixed with coat mutant RNA-stimulated product H^3-labeled. Incorporations were done in Su^+ extracts. Variation of electrophoretic mobility of *A* and *A'* is detected by the change in the ratio of H^3/C^{14} in the area around *A* and *A'*. In area *A*, this ratio is 0.55 H^3/C^{14}, and in area *A'*, it is 3.50 H^3/C^{14}. Spot *D* has a ratio of 1. Spot *A* has 699 cpm C^{14}. Spot *A'* has 716 cpm H^3. Spot *D* has 505 cpm H^3 and 508 cpm C^{14}.

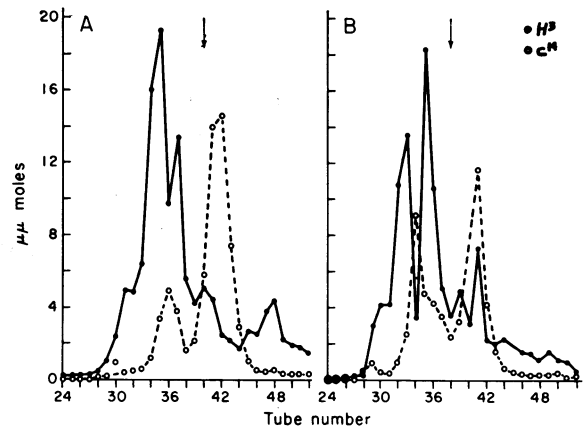


FIG. 3.—Elution profiles of soluble chymotryptic peptides of *in vitro* product from a Dowex-1 × 2 column. The C^{14} label is phenylalanine, and the H^3 label is serine. The arrow is the position of the reference tripeptide. (*A*) The elution profile of a chymotryptic digest of product stimulated by f2 RNA in Su^+ extracts. The labeled tripeptide is in tubes 40–44. (*B*) The elution profile of a digest of product stimulated by coat mutant RNA in Su^+ extracts. The labeled tripeptide is in tubes 39–42.

Suppressing action of sRNA: Since the nonsense mutation is in the N-terminal tryptic peptide of the coat protein, only a small fraction of the protein should be formed under nonsuppressing conditions. Therefore, a significant increase in net protein synthesis might be expected from suppression. In an Su^- extract, stimulation by mutant RNA of incorporation of C^{14}-lysine into acid-precipitable material is about tenfold less than the stimulation by wild-type RNA. The TCA-precipi-

table products stimulated by the mutant and the wild-type RNA show the same general tryptic and chymotryptic peptides, with one difference: chromotryptic peptide A' is present in place of peptide A in the product from mutant RNA. The low level of normal protein produced by mutant RNA in the Su^- extracts indicates either a low level of suppression or, possibly, an ambiguous reading of the nonsense triplet.

The effect of added sRNA on C^{14}-lysine incorporation in Su^- extracts containing mutant phage RNA is shown in Figure 4.

The results indicate that the sRNA from Su^+ bacteria markedly stimulates incorporation, in contrast to the lack of response to added sRNA from Su^- bacteria. The addition of Su^- sRNA to Su^+ extracts does not affect incorporation with mutant phage RNA. It is of interest that addition of Su^+ sRNA to Su^+ extracts with mutant phage RNA enhances incorporation, indicating that the Su^+ extract is limited in the amount of sRNA required by the mutant phage RNA.

In contrast to the stimulating effect of added Su^+ RNA on incorporation with mutant phage RNA in both Su^+ and Su^- extracts, no such effect is observed with wild-type phage RNA. Thus, the results in Figure 4 appear to provide a specific assay for suppression.

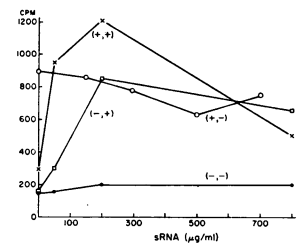


FIG. 4.—The effect of sRNA from suppressed (Su^+) and nonsuppressed (Su^-) bacteria on coat mutant RNA-directed incorporation using extracts of suppressed (Su^+) and nonsuppressed (Su^-) bacteria. The ordinate represents C^{14}-lysine-labeled TCA-insoluble material stimulated by coat mutant RNA. The Su^+ or Su^- extract used is identified by the plus or minus on the left between the parentheses. The Su^+ or Su^- sRNA used is identified by the plus or minus on the right between the parentheses. The points represent the amount of isotope incorporated after 40 min incubation. Each point is taken from a complete kinetic assay and was proportional to both the rate and final amount of protein synthesized in that experiment. In the (+, −) experiment, the original Su^+ extract was supplemented with 50 μg of Su^+ RNA.

Another possible assay for suppression involves the chain-terminating effect of a nonsense mutation under nonsuppressing conditions, resulting in the formation of a polypeptide fragment.[3] The presence of a fragment of the coat protein in Su^- extracts containing mutant phage RNA was examined in the experiment of Figure 5*A*, in which the C^{14}-phenylalanine-labeled product was passed through a column of Sephadex. A control for the experiment consisted of adding H^3-phenylalanine-labeled product from an Su^- extract containing wild-type phage RNA. The elution pattern of radioactivity has a combined C^{14} and H^3 peak in the excluded volume, which consists primarily of completed coat protein; it contains all of the expected coat peptides. In addition, there is a peak which elutes later and, therefore, represents a smaller component. This peak is completely absent in the H^3 profile. The material in the second peak runs as a single component at various pH's during electrophoresis on cellulose acetate. Chymotryptic fingerprints of this material yield peptide D but not A', suggesting that the peptide is a fragment of the coat protein.

Further evidence that the fragment seen in Figure 5*A* is correlated with the presence of a nonsense triplet is that it can be suppressed. Figure 5*B* shows an elution profile of C^{14}-phenylalanine-labeled product from an Su^+ extract containing mutant phage RNA, in combination with H^3-phenylalanine-labeled product from an Su^- extract also containing mutant RNA. The relative amount of fragment (late

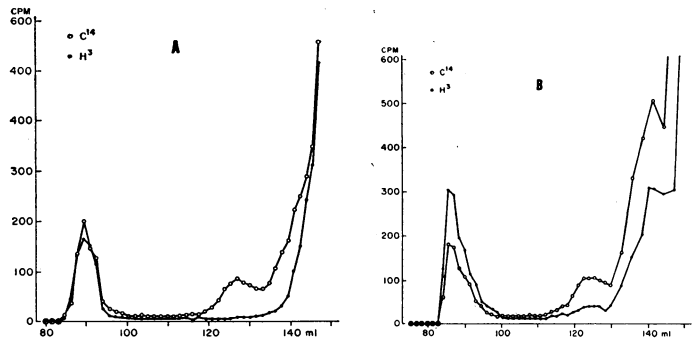


FIG. 5.—Gel-filtration of acetic acid-soluble products of *in vitro* protein-synthesizing systems. The radioactive amino acid incorporated was phenylalanine. For both these runs, approximately equal amounts of H^3-labeled and C^{14}-labeled TCA-precipitable material was added to the column. Each 300 cpm of C^{14} is equivalent to 1 $\mu\mu$mole of C^{14}-labeled phenylalanine, while each 150 cpm of H^3 is equivalent to 1 $\mu\mu$mole phenylalanine. (*A*) A comparison of wild-type RNA-stimulated product (H^3-label) and coat mutant RNA-stimulated product (C^{14} label) in Su^- extracts. The "coat fragment" elutes between 120 and 135 ml. (*B*) A comparison of coat mutant RNA in Su^+ extracts (H^3 label) and in Su^- extracts (C^{14} label). The "coat fragment" elutes between 120 and 130 ml. The Su^+ extract was supplemented with 50 μg of Su^+ sRNA.

peak) is about five times greater in the Su^- extract than in the Su^+ extract. The addition of optimal amounts of Su^+ sRNA to Su^- extracts reduced the fraction of fragment threefold.

It is to be expected that suppression by Su^+ sRNA in an Su^- extract would cause a substitution of serine for glutamine in chymotryptic peptide *A*. Fingerprints of the material from an excluded peak such as that shown in Figure 5*B* show a peptide corresponding to the position of the serine containing tripeptide (see Fig. 2). This agrees with the preceding results showing that serine is incorporated into the tripeptide when the mutant phage RNA is added to an Su^+ extract (see Fig. 3).

Discussion.—The data presented demonstrate that suppression of a nonsense mutation in an RNA phage can be achieved *in vitro* by addition of sRNA from an Su^+ bacterial strain to an extract of an Su^- strain. Suppression *in vitro* corresponds accurately to the effects obtained *in vivo:* a serine residue is inserted into the coat protein of the phage at the position specified by the nonsense triplet. Furthermore, in the absence of suppression, a fragment of the coat protein is produced *in vitro* which terminates at the same position.

It is noteworthy that chain termination induced *in vitro* by the nonsense triplet is not completely effective; in an Su^- extract, the mutant phage RNA is capable of specifying about 20 per cent as much complete protein as in an Su^+ extract. The resulting protein appears to contain serine in place of glutamine at the expected position. Thus, translation of the nonsense triplet is to some extent possible in the absence of added materials. For this reason, a net increase in the synthesis of complete protein *per se* cannot be used as the sole evidence for suppression, since it could result from an increased rate of chain initiation as well as a decreased rate of chain termination. The critical parameter is the ratio of fragment to complete chains. The present experiments show that this ratio markedly decreases upon addition of sRNA from an Su^+ strain. Further aspects of these problems will be developed in subsequent communications on chain initiation and chain termination.

The present results are in agreement with those of Capecchi and Gussin who used a similar phage assay system for *in vitro* suppression.[8] It remains to be shown whether the Su-1 gene determines the structure of a serine sRNA directly or indirectly, perhaps by specifying an enzyme which modifies a pre-existent sRNA.

* Supported in part by grants from the National Science Foundation.

[1] Benzer, S., and S. Champe, these PROCEEDINGS, **48**, 1114 (1962).
[2] Garen, A., and O. Siddiqi, these PROCEEDINGS, **48**, 1121 (1962).
[3] Sarabhai, A. S., A. D. Stretton, S. Brenner, and A. Bolle, *Nature*, **201**, 13 (1964).
[4] Nathans, D., G. Notani, J. H. Schwartz, and N. D. Zinder, these PROCEEDINGS, **48**, 1424 (1962).
[5] Notani, G. W., D. L. Engelhardt, W. Konigsberg, and N. D. Zinder, *J. Mol. Biol.*, **12**, 439 (1965).
[6] Weigert, M. G., and A. Garen, *J. Mol. Biol.*, **12**, 448 (1965).
[7] Stretton, A. D., and S. Brenner, *J. Mol. Biol.*, **12**, 456 (1965).
[8] Capecchi, M., and G. Gussin, *Science*, **149**, 417 (1965).
[9] Schwartz, J. H., these PROCEEDINGS, **53**, 1133 (1965).
[10] Zubay, G., *J. Mol. Biol.*, **4**, 347 (1962).

---

*CELL TURNOVER IN MAMMALIAN TISSUES: USE OF CELL DEPLETION MEASUREMENTS TO CALCULATE X-RAY REPRODUCTIVE SURVIVAL CURVES IN VIVO**

BY THEODORE T. PUCK

DEPARTMENT OF BIOPHYSICS, UNIVERSITY OF COLORADO MEDICAL CENTER, DENVER

Communicated October 4, 1965

In previous papers it has been shown that the single cell survival curves of mammalian cells can be quantitatively applied to the interpretation of the action of ionizing radiation on mammals.[1, 2] *In vitro* studies demonstrated that cells taken from a wide variety of mammals, and originating in different tissues, exhibited a mean lethal dose, D^0, of approximately 100 rads and a survival curve hit number usually lying between 1 and 3. Studies from several laboratories demonstrated that the survival curves of mammalian cells *in vivo* are similar to those *in vitro*. (The sensitivities may not be identical because of changes in tissue oxygenation and possibly other factors that could introduce differences of 20–140%.) Hence, it was proposed that reproductive death constitutes the primary action of irradiation with doses below 2,000 rads. Studies were undertaken in the bone marrow, spleen, and thymus of young adult mice quantitating the well-known cell depletion, which follows exposure to ionizing radiation. Tests were devised to determine whether the resulting cell loss was due only to the inhibition of cell reproduction, or whether cell destructive and evacuative processes were also initiated or accelerated in the affected tissues. A variety of tests gave results which appeared to indicate that reproductive inhibition alone is responsible for all or most of the observed depletion of nucleated cells, during the early period (less than 48 hr) following X irradiation. These tests included demonstration that after a limiting dose is reached, the time course of cell depletion remains constant, regardless of further increases in dose; and that agents like Colcemide and Vinblastine (Velban, Eli Lilly