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MASTER

TRIENNIAL PROGRESS REPORT:
BIOLOGICAL EFFECTS OF IONIZING RADIATION AT
THE MOLECULAR, CELLULAR AND ORGANISMAL LEVELS

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Triennial Progress Report
for work completed in FY78-80

Abstract

Two major accomplishments have been achieved in the past three years with the support of this contract. Firstly, the original Zimm theory of rotor speed dependent DNA sedimentation has been tested quantitatively and found to be correct, i.e., T4c and T4D+ DNAs sedimented with $S^{0}_{20,w}$ values as predicted by the equation of Zimm and Schumaker. Furthermore, the quantitative validity of the theory means that the size (M_r) of a DNA sedimenting under speed-dependent conditions is not undefinable but rather can be uniquely obtained by the application of that theory to the data. Secondly, the viscoelastic recoil (Γ_{11}), or more accurately, the zero shear rate reduced recoil ($\Gamma_{11,r,0}$) has been shown to be a quantitative direct function of the number of intact (T4c) DNA molecules present (per ml) in solution. This demonstration made possible the measurement of a direct survival curve for intact DNA molecules (i.e., without double-strand breaks) after exposure to ionizing radiation. A D_{37} of 47.4 krad was obtained for the DNA of T4c coliphage irradiated in air as a solution of phage particles. It is noteworthy that this survival curve measures the number of intact DNA molecules, not the average number of breaks/molecule.

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I Research Accomplishments

A. DNA Size, Organization and Repair

1. Size Measurement by Means of the Ultracentrifuge.

- (a) Re-examination of the Effects of Glucosylation and of Hydroxymethylcytosine on DNA Sedimentation Rate.

Since the T_{even} bacteriophage DNAs are the largest naturally occurring uniform size DNAs which can be readily and have been accurately measured by electron microscopy, they have been universally used as standards for DNA sedimentation. However, they all naturally have cytosine replaced by hydroxymethylcytosine and to varying extents are heavily glucosylated. If these substitutions affect the sedimentation of these DNAs, then the exponent of the S vs. M relationship must be corrected for these effects. Previously, the evidence (Lange *et al.*, 1977) had suggested that the use of T_4 DNA does not present a problem. However, more recent data of ours (Clark and Lange, 1980) shows that there is after all a problem. In collaboration with the laboratories of Dr. John Wiberg who has supplied us with $T4D+$ (hydroxymethylcytosine substituted (HMC+), glucosylated (G+)), $T4G-$ (a nonglucosylated version of $T4D+$, i.e., HMC+G-), $T4G+$ (a glucosylated version of $T4G-$ to test for reversibility of the glucosylation and for possible DNA deletions, i.e., HMC+G+), $T4c$ (a normal cytosine DNA lacking the hydroxymethylcytosines, i.e., HMC-G-) and $T4cp$ (a petite form of $T4c$) bacteriophage stocks, and of Dr. Tom Broker of Cold Spring Harbor who has helped us with the measurement of these bacteriophage DNAs on the electron microscope, we proposed to determine the sedimentation rates on Noll gradients (Noll, 1967) of each of these DNAs, T_2 and T_6 DNAs, and to compare the effects of the DNA substitutions on their sedimentation rates.

The lengths of all of the phage DNAs ($T4D+$, G-, G+/-, c, cp, $T2H$ and $T6$) have been determined relative to phage λ DNA by electron microscopy in our laboratory (using Dr. J. Maniloff's electron microscope). The length of λ DNA is known relative to that of $\phi X174$ DNA and the latter is known absolutely (in base pairs) from having been sequenced (Sanger *et al.*, 1977). Thus the length of λ , and therefore of all of the T_{even} series DNAs we have measured is known with considerable absolute precision.

A paper on these length measurements for non-glucosylated, unsubstituted, i. e., cytosine containing, DNAs (T7, T4cp, and T4c) and their sedimentation behavior has been published (Clark et al., 1980). This paper also derives $S_{20,w}$ vs. M equations based on T7, T4cp and T4c DNAs and the Gray-Bloomfield-Hearst Theory and shows that the use of T4D+ instead of T4c changes the exponent and coefficient values considerably.

- (b) Testing of the Zimm theory of rotor-speed dependent sedimentation.

As outlined in the renewal proposal background, a quantitative critical test of Zimm's theory had not yet been performed, and the Rubenstein and Leighton data indicated the possibility of a tube composition effect. We have now performed the critical test of the Zimm theory for swinging bucket rotors. Our conclusions (cf. Clark and Lange, 1980) are that provided: (1) the Cohen and Eisenberg (1968) apparent specific volume ($\bar{v}'$) correction is used instead of the partial specific volume ($\bar{v}$), so that $S_{20,w}^0$, the actual zero concentration S value (corrected to 20°C and water) in the gradient solvent at a given salt molarity, is used, and (2) the $S_{20,w}$ value is corrected for DNA solvation (Nieuwenhuysen (1979)) (the changing concentration of sucrose down the gradient effects the DNA hydration and this correction takes that into account), then the Zimm theory equations (1974) (or specifically, the form derived by Zimm and Schumaker (1976)), are quantitatively correct for both polyallomer (non wettable-hydrophobic) and nitrocellulose (wetable-hydrophilic) tubes. No evidence of a tube composition effect was found between these two tube materials provided that the tube wall thickness differences were taken into account, i.e., the volumes of gradients were different for the two tube types even in the same rotor buckets.

Thus DNA demonstrating speed dependent sedimentation is no longer unmeasurable at g forces (rotor speeds) which produce such behavior, but rather the speed dependent behavior yields a unique size value (M_r) for that DNA and in addition confirms whether or not the $S_{20,w}^0$ vs. M_r equation used is valid!

The main remaining problem is that the original Zimm theory contained an error (Zimm and Conrad, personal communication) and the corrected (i. e., now theoretically correct) theory does not agree with observation. One possible explanation is that the corrected theory might be correct for radial sedimentation (i.e., in zonal rotors) but that the non radial sedimentation in swinging bucket rotors causes DNA concentration changes at the tube walls and it is this concentration effect which causes the difference between theory and swinging bucket rotor obtained data. Another possibility is that a second cancelling error, as yet undiscovered, remains nested

within the theory and its correction will again yield the original predictions which we confirmed in the swinging bucket rotor. The zonal rotor test should distinguish between these possibilities.

Nevertheless, the original theory did correctly describe the behavior of T4c DNA in swinging bucket rotors, and therefore should be applicable to other DNAs (although with caution for $> 10^5$ dalton molecules).

Work is continuing in our laboratory to test the Zimm equations in the zonal rotor and test whether or not there is a tube wall DNA concentration effect.

2. Size Measurement by Means of Viscometry.

Re-examination of the Effects of Glucosylation and of Hydroxymethylcytosine on DNA viscosity.

Initial attempts were made to measure the viscosities of the T_{even} series (including glucosylation and cytosine variants) DNAs in the viscoelastometers, because the low shear stresses available in that device would have been near ideal. However rotor velocity was found to be quite sensitive to rotor vertical position (depth), presumably because of inhomogeneities in the induced magnetic drive field. Modified coils were constructed to alleviate this problem but lateral drift limited relative viscosity (η_{rel}) determinations to a precision of $\pm 3\%$. Therefore we returned to the use of the Zimm-Crothers low shear viscometer for viscosity determinations (precision typically $\pm 0.4\%$). With this apparatus we are now determining the relative viscosities (η_{rel}), the specific viscosities (η_{sp}), the reduced specific viscosities (η_{sp}/c) and the intrinsic viscosities ($[\eta]$) for the above described T_{even} series phage DNAs.

The results of section A 1 and A 2 (this report), should enable us to determine S vs. M and $[\eta]$ vs. M equations for normal, native, unmodified DNAs and to determine whether or not the subtractive or additive constants in the M vs. S and $[\eta]$ equations are significantly different from zero. In fact, they are, and we are now trying to get precise values for them.

3. Size Measurement by Means of Viscoelastometry.

The calibrations are still in progress (see section 1 f of the FY 78 proposal); the data processing programs have been thoroughly tested and are working reliably. However, in order to measure DNAs of T7 size accurately, a faster computer (e.g. Tektronix 4052 or 4054) will be needed. This will also be a requirement for the simultaneous operation of both viscoelastometers. Additional measurements of the principle retardation time (τ_{11}) for L5178Y mouse leukemia cell DNA yielded τ values comparable to those obtained previously by Dr. Lange in Dr. Zimm's lab. Some preliminary measurements of mercaptoethanol treated L5178Y cell DNA were made and the range of τ values obtained are consistent with the expected size of DNA subunits postulated to arise from the cleavage of protein linkage disulphide bonds. Calibrations of τ_{11}

vs. M_v using T4c and T4cp DNAs in 0.1 M salt are in progress. Ideally, T7 and fowl pox virus DNAs should also be included in the calibrations to make the L5178Y subunits size determination most reliable.

4. Measurement of Direct DNA Survival Curves by Means of Visco-elastometry.

The viscoelastic recoil of shear-stressed DNA molecules is most sensitive to the largest molecules present in the solution. If only a single molecular species (DNA size) is present in a creep-recovery experiment, the semilog plot of the relaxation recoil decay will be a single straight line with negative reciprocal slope, τ_{11} , and extrapolated intercept of this slope to zero recoil time, the recoil amplitude, Γ_{11} . If this monodisperse population of DNA molecules is irradiated, theory would lead us to expect that those molecules which received double strand breaks (DSBs) are removed from the population of molecules with relaxation decay rate constant τ_{11} , and the Γ_{11} intercept value should decrease denoting fewer such full-size molecules present. A quantitative test of this part of the theory has not yet been performed. As a part of this test, Dr. Lange has measured the values of τ_{11} and Γ_{11} for a series of T4c DNA solutions of various (10-25 $\mu\text{g}/\text{ml}$) DNA concentrations. The Γ_{11} values are functions of the average shear rate (κ) and relaxation time constant (τ_{11}), so by division by $\kappa\tau_{11}$, they are converted to a dimensionless parameter, the reduced recoil amplitude, $\Gamma_{11,r}$. Bowen (Ph.D. thesis, 1977) has shown that the logarithm of the reduced recoil amplitude, ($\Gamma_{11,r}$), has a linear regression on the quantity $\ln(1 + 3(\kappa\tau_{11})^2)$. The value of $\Gamma_{11,r}$ at $\kappa=0$ is the zero shear rate corrected reduced recoil amplitude, $\Gamma_{11,r,0}$. Dr. Lange has determined the value of $\Gamma_{11,r,0}$ for each of the DNA concentration solutions described above, and found a highly predictive regression of $\Gamma_{11,r,0}$ on DNA concentration (c) ($\mu\text{g}/\text{ml}$ or number of DNA molecules of full size/ml). This regression was $\Gamma_{11,r,0} = (0.093 \pm 0.006) + (0.0406 \pm 0.0003)c$, with $S_{y,x} = 0.004$.

When T4c coliphage were irradiated with ^{60}Co - γ rays (at DNA concentrations of 25 $\mu\text{g}/\text{ml}$) and their DNA subsequently released by heating (58°C) for 45 min in buffer containing 0.1% sarkosyl), the following values of $\Gamma_{11,r,0}$ were obtained: 0 rads, 1.107; 4662 rads, 1.125; 8189 rads, 0.943; 20,777 rads, 0.819; 37,272 rads, 0.566; 65,472 rads, 0.359. Thus by conversion of the $\Gamma_{11,r,0}$ values into intact molecule DNA concentrations (or numbers of intact molecules), a survival curve for intact T4c DNA molecules was obtained, with an inverse variance weighted least squares best fit (for simple exponential inactivation) of $D_0 = 47.4 \pm 2.3$ krad. These results were presented at the 6th International Congress for Radiation Research. The $\text{DNA } D_0$ obtained here, when divided by the $\text{pfu } D_0$ (plaque-forming assay) for irradiation in Air (41 krad), shows that 86.5% of lethality (to PFUs) is caused by DNA DSBs when irradiation is under oxic conditions. Okada (p 115, see Ref. 13 in the proposal) cites a personal communication from Freifelder that this ratio is 86% (based on analytical ultracentrifuge DNA data) for T4 (glucosylated) bacteriophage. For hypoxic (N_2) irradiation the ratio is 100% (see Renewal Proposal p 9, Section Aldii).

We have also obtained T4c plaque forming unit (PFU) survival curves for irradiation in Air (5 decades) and in Nitrogen (3 decades) under saturation radical scavenging conditions) and obtained, respectively, D_0 s of 41.01 ± 0.45 and 79.34 ± 1.99 krad yielding an oxygen enhancement ratio of 1.93. This is of interest because, based on our D_0 in Air of 41.01, and M_{-} values for T4c and T7 DNAs, we predict a $PFU D_0$ in Air for T7 of 176 ± 7 krad which agrees very well with the 175 krad value reported by Freifelder (1965). However, his irradiation conditions included a 2 minute gassing with N_2 prior to sealing in glass ampoules for irradiation and thus his value might be supposed to be for hypoxic irradiation. We also found that unless the liquid was thoroughly deoxygenated (for ca. 1 hr by vigorous bubbling of N_2 gas through it) and extreme care was taken to remove gas phase O_2 (by repeated vacuum and N_2 flushing), followed by sealing under N_2 gas flow) the PFU survival obtained was the same as for irradiations in Air. Therefore, the Freifelder (1965) $PFU D_0$ value for T7 is likely to be an oxic value. The predicted (from our T4c hypoxic $PFU D_0$ and the M_{-} values) hypoxic T7 $PFU D_0$ value is 348 Krad.

We are in the process of obtaining replicates of the T4c intact DNA survival experiment (in the same radical scavenging buffers as used in the PFU survival experiments) so as to test whether or not the lethal event for PFU survival is thus induction of a single DSB. These experiments are being done with irradiations, both in Air and under hypoxia to test for an oxygen effect for DNA DSBs (presently there is not thought to be one, but if the Freifelder data were in fact not truly hypoxic, then the methods used in contemporaneous experiments reporting no oxygen effect may have yielded comparisons between two oxic conditions). Such data for T4c coliphage should provide a sound basis for confidence in the results of similar experiments on DNA subunits.

5. Test of the Universality of the DNA Unit and Subunit Organization of the Mammalian Chromosome.

Culture of the Indian Muntjac Cell

Muntjac (*Muntiacus muntjac*) cells, chosen for their low chromosome number ($2n=7$; 4 autosomes plus XYY) and wide separation in chromosome sizes, are now maintained in monolayer in our laboratory. The rather slow growth rate of these cells ($T_2 \sim 40$ hrs) makes it difficult to obtain the large number of cells needed to obtain reasonable quantities of separated chromosomes. Therefore we have been experimenting with ways to grow more cells per ml of medium and in particular have been developing a 3-dimensional growth technique where the cells grow attached to polyacrylamide beads. The cells do not grow at all well in suspension but the polyacrylamide bead attachment method should yield high surface areas for growth with relatively small medium requirements. Experiments are still in progress.

B. Sublethal Damage Repair

1. Cell Cycle Stage Dependence of Toxicity of Specific Metabolic Inhibitors.

Dr. Lorraine E. (Hatch) Sheck (under Dr. Lange's supervision) has completed and successfully defended her Ph.D. thesis on "The inhibitory and toxic effects of methylmercuric chloride (MeHgCl) on the cell cycle of cultured mammalian cells" and is now preparing for publication some 8-10 papers based on her thesis. Although MeHg is toxicologically interesting per se, it was also of interest to us in this study because of its known binding to DNA and possible binding to DNA linkage proteins. However, there seems also to exist a membrane activity of MeHg so that it has not yet been possible to interpret its inhibitory and toxic effects solely in terms of DNA metabolism. The titles of the in preparation papers are listed under "Publications (IV)." A paper on the "Hydroxyurea-induced progression effects on HeLa G₂ cells" was presented at the 13th Annual Meeting of the Tissue Culture Association, and is in press with Cell and Tissue Kinetics (Sheck and Lange, 1980).

C. Organismal Level Effects (Planarian Study)

1. The Mechanism of Tissue Polarity Control.

Several papers based on Dr. V.E. Steele's doctoral thesis (under Dr. C.S. Lange) have been published. That work demonstrated the following points: (1) the active agent of Lender's cell-free extract which inhibits differentiation of stem cells to form brain tissue, is a highly electronegative protein with a PI (isoelectric point) between 4.78 and 5.38 and a molecular weight between 2 and 4×10^5 daltons; (2) that this protein can readily be electrophoresed in vitro and probably, also, in vivo; (3) that this protein with its normal production by the brain and an indicated flow towards the posterior, could be the means for maintaining the planarian's normal tissue organization and polarity, and that a cut (lesion) interrupting this flow could give rise to local depletion of the inhibitor at the anterior surface of the posterior piece thus permitting brain formation (head regeneration) there but inhibiting it elsewhere throughout the animal; (4) radiation doses can inhibit the ability of stem cells (reproductively dead) to respond to the differentiation stimulus (loss of differentiative integrity); and (5) these results are of broad general interest because the same effects (1-3) are obtained (Lender and Deutch (1961)) with nine-day chick embryo brain extracts. The effects of external electrical fields or stimuli and of nerve rerouting in amphibians and mammals (Becker and Spadaro, Bull. N.Y. Acad. Med., 48, 627 (1974)) strongly suggest that similar mechanisms apply in higher animals and, perhaps, in man.

A subsequent paper by Lange and Steele, which appeared in Differentiation (11, 1-12 (1978)), presents our bioelectric-electrophoretic model for the control of anterior-posterior tissue polarity and tests two major predictions of our model. The predicted results would not have been expected on the basis of previous models (or theories) and were shown to be confirmed by the experimental data. The confirmed predictions were that there existed an in vivo bioelectric field with the brain (cerebral ganglia) as the cathode and the caudal parenchyma as anode. The bioelectric field was found to exist both in the nervous tissue (always negative relative to ground but decreasingly so caudad) and in the parenchyma (always positive relative to ground but increasingly so caudad) and to be of the correct polarity to act as the motive force for electrophoresis

of the negatively charged brain differentiation inhibitor protein from its source (the brain) to the distal caudad regions of the parenchyma. The bioelectric field was also found to exist essentially undiminished after decapitation of the animal, thereby providing the motive force for the removal of inhibitor protein molecules from the anterior wound surface.

Current work (Lange, Biophys. J., 25, 82a (1979) and Fed. Proc., 39, 1614 (1980)) has shown that the above model can be restated mathematically, the equations solved, and time course and inhibitor concentration distributions predicted.

II Research Objectives

The overall long-term aim of our research program has been and is to make possible the explanation of organismal events at the cellular level (e.g., using the planarian as a model system) and to make cellular events explicable at the molecular level. With these broad aims in mind we intend specifically to continue our work, a) at the molecular level to determine the packaging and structure of chromosomal eukaryotic DNA and its breakage and repair by external agents (such as ionizing radiation); b) at the cellular level to correlate specific metabolic events (or blockages) with DNA repair (or failure to repair; and c) at the organismal level to test further our models of polarity maintenance and of ageing. We are proposing, in the renewal of this contract, to use T4c coliphage as a probe for *E. coli* repair capabilities. Dr. Lange has already produced, by transduction, a *recA*⁻ version of the host cell line used to grow T4c, so repetition of the PFU and DNA experiments of section A4, should permit a test of the Resnick (1976) Model for DSB repair. A direct test in *E. coli* DNA has so far proven unreliable since published methods have yielded either fragmented (ca. 10⁸ dalton) or nonlinear, collapsed, DNA for untreated control cells. Furthermore, since the viscoelastometer has successfully yielded results, for T4c DNA which are in agreement with sedimentation derived results, the application of viscoelastometric methods to study repair in larger DNA molecules, where the advantages of viscoelastometry become most apparent, should proceed with considerable confidence.

III Training of Graduate Students

A. Research Students

1. Postdoctoral tenures

Dr. R.W. Clark has successfully completed three years as Instructor (Research) in Dr. Lange's lab and has been promoted to an assistant professorship in this department.

2. Ph.D. students

- a. Dr. Lorraine E. (Hatch) Shack has successfully defended her Ph.D. thesis in Toxicology on work she performed under Dr. Lange's supervision and in his laboratory. She examined in particular detail the cell cycle stage dependent toxic effects of methylmercury. She now holds an NIH post doctoral fellowship (on mercury toxicity) here in Rochester (working jointly in Dr. M.W. Miller and Dr. C.S. Lange's labs).

- b. Dr. Ying Kai Fu has successfully defended his Ph.D. thesis in Radiation Biology on work he performed under the joint guidance of Drs. M.W. Miller and C.S. Lange on the toxic effects of 1MHz ultrasound. Dr. Fu has returned to teach and do research at the Chung-Shan Institute of Science and Technology in Taiwan, China.
- c. Donald E. Newmeyer spent a year working in Dr. Lange's lab, preparing for his doctoral research for a Ph.D. in Biophysics.
- d. Patricia S. McElrath, a Biology Graduate from Williams College, spent a year in Dr. Lange's laboratory as a Technical Associate, to gain laboratory experience in a biomedical research field before continuing her studies at Medical School.

B. Undergraduates and High School Students

- a. Sophia Chan, an undergraduate chemistry major at the University of Rochester spent a year (1977-78) in Dr. Lange's laboratory preparing glassware, making solutions, and learning how a DNA biochemistry and cell culture laboratory is run.
- b. Daniel Alley, an undergraduate biology major at the University of Rochester, spent a year (1978-79) in Dr. Lange's laboratory preparing phage stocks, doing plaque-forming assays, and running experiments on the viscoelastometer.
- c. Vincent Tritto and George Tyrian, undergraduate chemistry majors at the University of Rochester each spent half a year (1979-80) in Dr. Lange's laboratory, preparing glassware, solutions, media, phage stocks, doing plaque-forming assays, and running experiments on the viscoelastometer.
- d. Astrid Borgstedt, a 3rd year high school student (first place winner of her local school science fair) spent the summer as a volunteer working with the planarians and learning about their electrophysiology.

IV Publications

1. C.S. Lange, D.F. Liberman, R.W. Clark, and P. Ferguson (1977), The organization and repair of DNA in the mammalian chromosome. I. Calibration procedures and errors in the determination of the molecular weight of native DNA, Biopolymers, 16, 1063-1082.
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17. L.E. Sheck and C.S. Lange (1980), The effects of hydroxyurea on the progression of HeLa G₂ cells into mitosis, Cell and Tissue Kinetics (in press).
18. C.S. Lange (1980), Polarity control and predicted organizer protein concentration vs. time relationships in the transversally sectioned planarian, Federation Proc., 39(6), 1614(#72).
19. C.S. Lange (1980), Viscoelastometric determination of a radiation survival curve for intact DNA molecules, Radiat. Res., (in press).
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21. C.S. Lange (1980), On the use of the zero shear corrected reduced viscoelastic recoil amplitude to measure the concentration of largest DNA molecules, (in preparation).
22. C.S. Lange (1980), A survival curve for intact DNA molecules determined by viscoelastometric recoil, (in preparation).
23. L.E. Sheck, C.S. Lange and M.W. Miller, Initial effects of methylmercuric chloride on the cell cycle of mouse leukemic cells (L5178Y); dose-dependent induction of growth blocks within the life cycle, (in preparation).
24. L.E. Sheck, C.S. Lange and M.W. Miller, Characterization of a specific S-phase effect on mouse leukemic cells (L5178Y) during low dose treatment with methylmercuric chloride, (in preparation).
25. L.E. Sheck, C.S. Lange and M.W. Miller, Mitosis-related loss of viability in mouse leukemic cells (L5178Y) treated with high doses of methylmercuric chloride, (in preparation).
26. L.E. Sheck, C.S. Lange and M.W. Miller, Restoration of reduced exponential growth rates of mouse leukemic cells (L5178Y) during prolonged exposure to methylmercuric chloride; identification of a dose-dependent lengthening in specific cell cycle phases, (in preparation).

27. L.E. Sheck, C.S. Lange and M.W. Miller, Adaptation and resistance of subpopulations of mouse leukemic cells treated with lethal doses of methylmercuric chloride, (in preparation).
28. L.E. Sheck, C.S. Lange and M.W. Miller, The effect of Methylmercuric chloride on the progression of HeLa G₂ cells into mitosis, (in preparation).
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