THE INFLUENCE OF TEMPERATURE UPON THE INACTIVATION OF A BACTERIAL VIRUS BY X-RAYS

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A discussion of the nature of the mechanisms of radiation damage in living systems requires distinction between so-called direct effects on sensitive sites or "targets," and indirect effects, by which the biological action is accomplished via some intermediate entity in the system such as a free radical produced directly by the radiation. One of the methods used to distinguish direct from indirect action consists in measuring radiosensitivity of the system while suspended in liquid media of different composition. In this manner one presumably can estimate the amount of direct action by comparing results obtained in pure water with those in some organic medium which, having high affinity for very reactive radiation-produced substances, should protect the cells from these intermediate substances. Such an approach has been used by a number of authors, a recent instance being Watson's study of radiation effects in the bacteriophages of Escherichia coli.\textsuperscript{1, 2}

It cannot be presumed, however, that changes in the outside environment allow discrimination of direct and indirect effects within the cell itself, or even within the virus particle, and for distinction on a subcellular and subparticle scale other methods have to be employed.

One method is a study of the effect of temperature on radiosensitivity. Above the very low temperature region temperature changes probably should not affect ionization and direct inactivation of a vital target, but temperature changes should affect the activity of any intermediate substance that affects secondarily the sensitive site, either by changing diffusion rates or by affecting reactivity itself, or by both methods.

In this investigation we have studied the effects of widely separated temperatures during x-irradiation on survival of the T1 bacteriophage of E. coli. These particles appear to be inactivated by single ionizations and they can be dried and carried to very low temperatures with little inactivation. In this dried state one is dealing with effects within the particle itself, and any separation of effect brought about by the temperature differences occurs within the virus.

A preliminary report has appeared previously.\textsuperscript{3}

Materials and Methods.—Suspensions in broth of T1 phage were dried in aluminum sample cups, irradiated in vacuo at controlled temperatures,
and assayed for survival on *E. coli*, strain B. The method of preparation involved distribution of 50 λ or 100 λ aliquots of broth suspensions of approximately 5 × 10⁸ particles per ml. as a drop at the bottom of each cup. The cup was an Aloe-Willett cap of 22 mm. outside diameter, modified at the bottom by a depression 14 mm. in diameter and 1 mm. in depth. The depression defined the maximum diameter of the drop. Such preparations were dried from the liquid state in a 160-mm. vacuum top desiccator, by evaporation with a Duo Seal Vacuum pump in a cold-room at 0–3°C. Evacuation was controlled to proceed in slow stages, with 3–4 hrs. elapsing in the first stage before the pressure was allowed to drop below 5 mm. Hg. Inactivation and loss associated with boiling in early stages was thus avoided. After the first stage, the pump was allowed to run continuously for 16–18 hrs., the pressure falling below 1 mm. Hg within the first hr. of this interval. This drying procedure resulted in less inactivation and less variability between replicate samples than any other method employed, including lyophilization. Nevertheless, the data from several experiments had to be rejected on account of excessive inactivation attributable to the drying process. In the four experiments reported here, survival through dehydration and rehydration was higher than 95%. The dried preparations were stored at 25°C., and were kept at atmospheric pressure except during irradiation and temperature exposure.

The irradiation apparatus included a 50-kv. Picker machine with a Machlett beryllium window tube operated at 40 ma. without additional filtration. A doughnut-shaped chamber with an internal bore of 20 mm. diameter was affixed to the head of the tube. At one end of the bore was the Be window and at the other end the sample cup, the irradiated sample being 10 cm. from the target of the tube. Evacuation of the chamber was effected through a side-arm connected to the bore and monitored by a manometer. Pressure was maintained at less than 0.1 mm. Hg throughout the exposure interval. Temperature at the bottom of the cup was monitored by an iron-constantan thermocouple located on its outer surface, while the entire sample cup was immersed in an appropriate isothermic medium within a Dewar flask. The isothermic media employed were liquid nitrogen (77°K.), solid carbon dioxide-acetone (176–180°K.), ice water (273°K.), and water (310°K.).

The dosimetry of the system employed has not yet been determined accurately. Variations in doses delivered were accomplished by varying the time of exposures, and all doses are expressed in terms of minutes. The best estimate of actual dosage is based on the manufacturer's estimate of approximately 62,000 r/min. at 10 cm. As noted below the results obtained agree very well with those of others.4

Immediately after irradiation, samples were restored to 25°C. and
atmospheric pressure. The phage particles were resuspended in broth, and dilution and plating were carried out in conventional fashion. The maximum time lapse between irradiation and plating was 4 hrs., and replicate samples from the beginning or end of this interval indicated absence of a standing effect when broth is the suspending medium. Effects of temperature and pressure changes per se, if present, were too small to be detected beyond the survival limits of the controls. In each experiment an average of 3 sample cups per point was employed, and titers were calculated from plaque counts of 5 plates per cup.

Results.—The relationship between treatment and survival at a particular temperature (Fig. 1) is considered exponential and the relation,

\[ \frac{N}{N_0} = e^{-kT D} \]  

appears to be valid, with dose (D) being in minutes of irradiation under the conditions described, and \( kT \) being the dose inactivation constant in reciprocal minutes at the absolute temperature. The numerical value of \( kT \) (± its standard error) at each temperature was estimated by a least squares approximation for each exponential line to be as follows: at 77°K. it is 0.115 ± 0.007; at 176°K. it is 0.128 ± 0.015; at 273°K. it is 0.180 ± 0.010; and at 310°K. it is 0.166 ± 0.014.

These values are plotted against temperature in figure 2. The type of response is not determined by the points, but assuming a linear relationship, we calculate that

\[ kT = 0.00027T + 0.091. \]
When this is substituted into expression (1), the survival of dried T1 phage is empirically described as

$$N/N_0 = e^{-D(0.091+0.00027T)}$$  (3)

when treated and measured under the conditions described herein.

It is important to note that this expression is known to be valid only for temperatures below 37°C. Even at 37°C. there is some thermal inactivation with time. For instance, at 35°C., T1 phage in our laboratory dried from broth is inactivated by about 10% over 24 hr.; at higher temperatures more rapid loss is certainly to be expected. Preparations held at 25°C. for 24 hrs. and at 35°C. for 4 hrs. were indistinguishable from others held at 0–5°C., for the same lengths of time. At the two higher temperatures, manipulations, as indicated above, were rapid enough to obviate the added complication of simultaneous inactivation from two treatments, heat in the higher ranges and x-rays, and no consideration is being given here to temperature results above 310°K.

The general empirical expression relating survival with dose and temperature indicates that at absolute zero there may be a real dose inactivation constant (the intercept of the line in Fig. 2). The latter extension has not, of course, been tested. Indeed the authors do not consider that 77°K. is close enough to predict with certainty that inactivation will proceed at absolute zero at the rate indicated, and there is need for tests at temperatures much closer to zero than it has been possible to make to date. However, the intercept indicated is a measure of the tempera-

![FIGURE 2](image-url)

**FIGURE 2**

The relationship between the dose inactivation constant and temperature for dried coliphage T1.
ture-independent radiation effect, and if it exists it alone is a measure of sizes or cross-sections of targets that can be believed to be directly affected by radiations.

Discussion.—Adams and Pollard report no apparent change in inactivation volume at temperatures between 203°K. and 310°K., but it should be noted that the spread of the points in their low temperature region together with the narrow temperature range tested mask an effect of the magnitude reported in this paper. The temperature effects on radiation sensitivity reported by them occur above 40°C., and being strikingly different in magnitude should probably be considered different in mechanism from those reported here.

In view of the small change with temperature, the possibility was considered whether the physical size of the target decreases as the particle contracts in the lower temperatures to an extent which accounts for the lowered efficiency of the x-rays. We are not aware of any measurements of change in volume of phage with temperature, but if we choose as an outside condition one of the highest values for coefficient of cubical expansion of organic materials with temperature cited (tar = 6–8 X 10^{-4} volume/unit volume/degree C.), over the range of 77 to 310 we calculate a volume increase for this material of only 18.6% (233 X 8 X 10^{-4}). It is unlikely that the virus particle physically expands with increasing temperature to this extent. Yet relative “target” size of the phage particle at 77°K. as compared with that at 310°K. calculated by comparing e^{-1} doses at the two temperatures (from equation 3) is 56% greater (8.9 min./5.7 min. = 1.56), nearly three times that which can be accounted for in terms of cubical expansion. Therefore, even if cubical expansion of the particle contributes to the effect, it seems unlikely that it is more than one of the minor reasons why a temperature rise results in increased radiation efficiency.

An analysis of the change with temperature in rate of inactivation by x-rays in standard chemical kinetic terms shows that the usual test for simple kinetics (logarithm of rate against reciprocal of temperature) using the Arrhenius formulation does not yield a straight line, nor, indeed, is such a result necessarily to be expected. Furthermore, in the absence of much more elaborate evidence, a thermodynamic and kinetic explanation critically demonstrating a mechanism or mechanisms is impossible.

However, a tentative model in kinetic terms is possible. Suppose that the inactivation constant is composed of two components, one temperature independent and one temperature dependent. The constant observed at any given temperature should, then, be separable into these two components by subtraction, and the value which when subtracted leaves residues which are in a first order Arrhenius relation \( (k' = e^{-\Delta H/RT}) \) with temperature is, according to this model, the inactivation constant of the
temperature independent portion. It was found that a straight line relation is approached as the value of the temperature independent portion of the constant approaches the value of the total inactivation constant at 77°K., and the "activation energy" indicated by the residues obtained in this way is about 1100 calories.

The temperature independent part of the inactivation constant may be thought of as the rate at which x-rays inactivate the virus particle by affecting directly the sensitive sites. But there are several conceivable meanings that could be attached to the temperature dependent portion of the inactivation constant. For instance, the \( \Delta H \) of about 1100 calories or some portion of it can represent the activation energy of some reactive compounds produced by radiation. The value estimated here is not out of line with the estimated values for free radicals. Or, the estimated constant may describe, at least in part, the effects of temperature on some diffusion mechanism. That such exist in the solid state is indicated by Mayburg and Lawrence\(^7\) who describe changes in conductivity of polyethylene after \( \gamma \)-irradiation at different temperatures and who calculate \( Q \) values as low as 3000 cal., representing to them the control by temperature of the migration of protons in this solid system. Other similar studies have been made on the relation between temperature and the development of latent photographic images.\(^8\) In biological systems a diffusion mechanism has been offered to explain the action of liquid nitrogen temperatures in modifying x-ray effects in plant seeds and pollen,\(^9\) with effectiveness of a given dose being reduced by 80% at the low temperatures.

The meaning of this interpretation in regard to exponential survival curves is that while the "single hit" interpretation is still allowed, the hit may occur very close to but not necessarily within the sensitive site itself. The intermediate substances formed are very reactive chemically as indicated by the low estimate of over-all activation energy, can migrate but short distances before reacting and removing themselves from the system, and, therefore, have very short lives. The consequence is an appearance at ordinary temperatures of temperature independence and direct action.

The data of course do not require the interpretation described above. Indeed, the model suggested by Adams and Pollard\(^4\) which demands the simultaneous breakage of several critical bonds can conceivably be made to fit. Furthermore, the possibility of competitive reactions with different energy requirements, recombinations within vital targets, or several combinations of these with the diffusion model are not eliminated. Our statement of the energy requirement is an over-all description that does not separate one possibility from any other.

Even without a clear physicochemical explanation, these results impose
a refinement on all considerations of target size in T1 coliphage deduced from x-irradiation data. Using our inactivation value for 310°K., and the rated dosage of the 50 kv.p. x-ray machine, we calculate the sensitive volume of T1 in our laboratory to be $2 \times 10^{-18} \text{cm}^3$, which agrees with the value reported by Adams and Pollard for the range 203° to 313°K. The results here indicate, however, that the value at very low temperatures may be one-half of that indicated by measurements at room temperature, and any consideration of the structure of phage based on x-irradiation data is subject to this restriction.

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**ELECTRON MICROSCOPY OF THE NUCLEIC ACID RELEASED FROM INDIVIDUAL BACTERIOPHAGE PARTICLES**

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It has been known for some years that certain bacterial viruses can be “shocked” osmotically by rapid dilution into distilled water of a suspension of virus in a concentrated salt solution. As a result of this treatment the suspension loses most of its virus activity, and becomes extremely viscous. When the mixture is subjected to differential centrifugation, instead of the typical virus particles normally observable in the electron microscope only flattened virus ghosts are found in the sediment. Herriott has shown that these ghosts are free of the nucleic acid which makes up some 40% of the intact virus, and are composed of protein. The ghosts retain the origi-