Peroxide Formation in Gamma Irradiated Citrate Buffer

In the course of our studies of radiation effects on solutions of deoxyribonucleic acid (DNA) of *E. coli* 15T⁻, we have observed peculiar post-irradiation changes in the UV-absorption spectrum of the irradiated solutions. For a period of 1–2 h immediately after irradiation the optical density (OD) in the region 240–280 nm of the solution was observed to regularly increase. This increase was proportional to the dose of cobalt-60 gamma rays employed. The radiation source was a Gamma cell 200 (Atomic Energy Commission of Canada, Ltd.). An even more dramatic effect was seen when these irradiated DNA solutions were heated in the Beckman DU spectrophotometer for the purpose of obtaining thermal denaturation curves using the usual techniques[1,2], as shown in Figure 1.

This result was obtained only if irradiation was carried out with oxygen bubbling through the solution. Substitution of nitrogen bubbling eliminated the post-irradiation increase in UV-absorption as well as the unusual thermal denaturation curves. This result suggested a peroxide was being formed either with the DNA or with the buffer ingredients. By a process of elimination it was demonstrated that only sodium citrate was involved in the post-irradiation reaction.

The standard buffer used in most DNA work is 0.15 *M* sodium chloride (NaCl) plus 0.015 *M* sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) in glass distilled water with a pH of 7.0[3,4]. Aqueous solutions of 0.015 *M* sodium citrate (Baker Analyzed Reagent Grade, reported to be 99.8% pure) were prepared in triple distilled water, and irradiated in a radiation cleaned Pyrex vessel supported in a Lucite jig at zero degrees. Either prepurified nitrogen or oxygen was bubbled through the solution for 30 min prior to and during irradiation. After irradiation, solutions were immediately placed in a quartz cuvette in a Beckman DB recording spectrophotometer, temperature controlled at 25 °C. The increase in absorbance at 260 nm as a function of time as well as the entire spectrum was recorded. Peroxide analysis was made by KI-starch titration. Heating experiments with irradiated solutions were performed in a Beckman DU spectrophotometer fitted with thermospacer plates connected to a Haake variable temperature heating bath. Temperatures were measured with special (Pyrocell) short-bulb thermometers immersed in the Beckman cell. Absorbed dose was calculated using conventional ferrous sulfate dosimetry with a G value of 15.5.

The change in absorption spectrum between 220 and 300 nm of 0.015 *M* sodium citrate plus 32 kilorads with time is shown in Figure 2. The appearance of a new product with an absorption maximum at 250 nm is seen, its concentration reaching a maximum approximately 1 h after irradiation. This compound remains stable at room temperature for at least two weeks. 1 h following irradiation the solution also gives a positive test for peroxide with KI-starch. The rate of increase in peroxide concentration parallels the increase in absorption shown in Figure 2. If nitrogen is bubbled through the sodium citrate solution prior to and during irradiation, no change in absorption spectrum is seen, and the KI-starch test is negative. We therefore believe that a product having the characteristics of a 'peroxy citrate' is formed, which absorbs in the UV-region of the spectrum, having an extinction coefficient of $3.8 \cdot 10^3$. The KI-starch titration experiments indicate that a dose of 32 kilorads results in approximately 1% conversion to this product, with a calculated G value of 4.5.

It only remained to explain the peculiar changes in UV-absorption upon heating irradiated solutions of DNA in saline-citrate buffer (Figure 1). A solution of 'peroxy

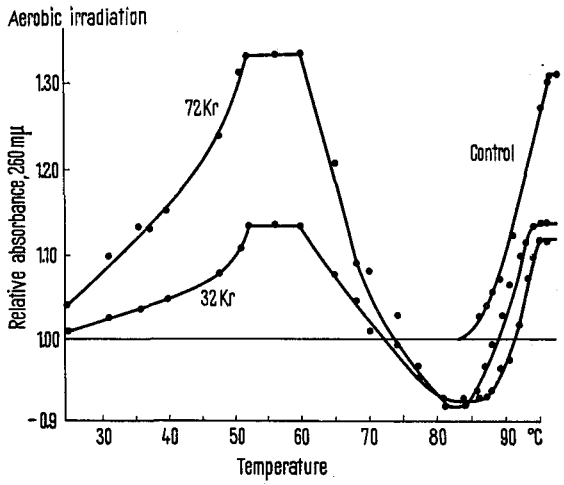

Fig. 1. Thermal denaturation curve of DNA from *E. coli* 15T⁻. Control, and irradiated in saline-citrate buffer at zero degrees C under oxygen bubbling conditions with 32 kilorads and with 72 kilorads.

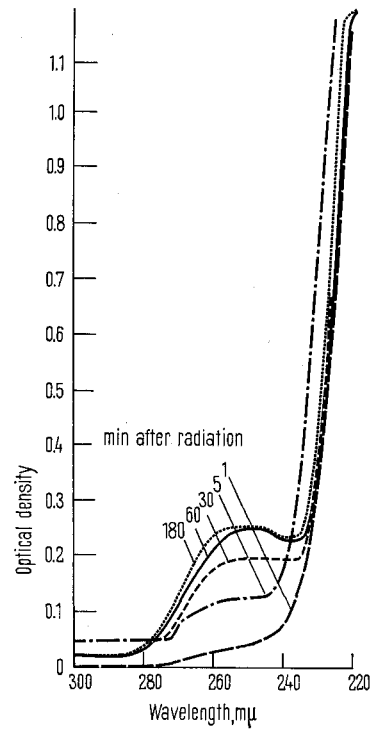

Fig. 2. Absorption spectra of 0.015 *M* sodium citrate irradiated with 32 kilorads under oxygen bubbling, measured at various times after irradiation.

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[2] J. MARMUR and P. DOTY, J. molec. Biol. *5*, 109 (1962).
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[4] F. HUTCHINSON, Radiat. Res. *14*, 721 (1961).

citrate' was prepared by irradiating 0.015M sodium citrate + 0.15M sodium chloride (32 kilorads) under oxygen bubbling and waiting 1 h. This solution was then placed in the thermostatted cell compartment of a Beckman DU spectrophotometer, and slowly heated. The absorbance at 260 nm increased very slightly as it was heated to 55 °C, apparently due to accelerated residual peroxide formation, since a portion kept as a control also increased slightly in absorption within two days, and then rapidly declined as the temperature reached 60–70 °C (Figure 3). This would be the expected range of decomposition of a per-acid, as observed with peracetic acid and persuccinic acid. The unusual hyperchromic effect of irradiated DNA solution shown in Figure 1 is therefore due to the formation and decomposition of the product formed in the irradiation of sodium citrate.

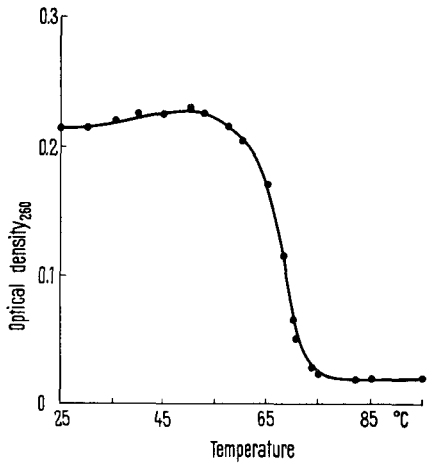

Fig. 3. Thermal decomposition curve of 'sodium peroxy-citrate' in saline citrate buffer.

The radiation chemistry of solutions of organic acids has been studied by others, and decomposition into hydrogen and carbon dioxide is generally observed. The yield for formic acid decomposition for example is G = 3.2[5], while acetic acid and glycolic acid give H_2 and dimeric products. Ascorbic acid in solution is oxidized by gamma radiation to dehydroascorbic acid with oxygen saturation enhancing the rate of destruction, but a post-irradiation reaction is not observed[6]. The reactive intermediate is presumably not a free radical but an oxygen containing species which undergoes a rearrangement to the peroxide.

In a separate experiment in which H_2O_2 was added to a 0.015M solution of sodium citrate, no increase in UV-absorption with time was observed. The results presented here indicate that a product of radiolysis of sodium citrate, a commonly used buffer material, is sodium 'peroxy citrate', formed during a post-effect. Oxygen must be present during irradiation for this product to form.

Résumé. Les résultats présentés ici suggèrent la formation d'un produit du type péroxide après l'irradiation, en présence d'oxygène, d'une solution aqueuse de citrate de sodium tamponnée au pH 7.

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Appearance of Pre-α_2-Globulins Soon After the Very First Dose of Diphtheria Toxoid in Horse

The changes in the serum protein pattern during one course of immunization of horse with diphtheria toxoid was measured by us. The electrophoretic separation of serum proteins of horse before commencement of immunization and four days after the first dose of 5 ml of diphtheria toxoid was carried out on agar gel at pH 8.6 according to the method of Giri[1] as modified by Acharya et al.[2]. The lipoproteins were analysed according to the method of Das and Giri[3]. A component with electrophoretic mobility between α_1- and α_2-globulin appeared soon after the first dose of diphtheria toxoid (Figure 1). We preferred to call this component pre-α_2-globulin. In addition to this, there occurred a slight decrease in albumin also soon after the first dose. There also appeared a new lipoprotein component with the electrophoretic mobility between α- and β-lipoprotein as seen in Figure 2. We preferred to call this component pre-β-lipoprotein. Since no detectable antibody activity was found at this stage in the α-globulin region, the increase in the pre-α_2-globulin soon after the first dose of diphtheria toxoid cannot be attributed to antibody formation.

Repeated injections at four day intervals for about two months showed no significant increase or decrease of pre-α_2-globulin, in spite of the marked increase in total proteins from 6.8 to 9.6 g per 100 ml, which was found to be mainly due to increased β- and γ-globulins. But a considerable amount of antibody activity (nearly 7–8% of total activity) was also found to be associated in α-globulin region after the hyperimmunization of horse, which agrees well with the previous observation made by Raynaud[4], and also by Rao[5]. The appearance of pre-β-

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